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"Pharmacological Ischaemic Preconditioning: Design and Synthesis of mito-K_{ATP} Channel Openers and Aldose Reductase Inhibitors"

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1 POTASSIUM CHANNELS

1.1 Introduction

Potassium channels are an ubiquitous group of ion channels involving in a multitude of physiological functions. They are multimeric integral membrane proteins forming transmembrane aqueous pores through which K^+ specifically permeates at a rate of 10^6-10^8 ions/s.[1] Many molecular subfamilies of K^+ channels are known and these correspond to the physiological signals by which pore opening is controlled such as voltage; Ca^{2+}; ATP; G proteins or polyamines, but a unique set of functional determinants characterize this superfamily of membrane proteins. These include (i) a water-filled permeation pathway (pore) that allows K^+ ions to flow across the cell membrane, (ii) a selectivity filter that specifies K^+ as permeant ion species, and (iii) a gating mechanism that serves to switch between open and closed channel conformations.[1]

K^+ channels are classified on the basis of primary amino acid sequence of the pore containing unit (α subunit) into three major families: the voltage gated (Kv) containing six-transmembrane-regions with a single pore; the inward rectifier (Kir) containing only two-transmembrane-regions and a single pore; the two-pore tandem K^+ channels containing four transmembranes with two pores. A tripeptide sequence Gly-Tyr(Phe)-Gly is common to the pore of all K^+ channels and constitutes the signature motif for determining K^+ ion selectivity. The "slowpoke" Ca^{2+}-activated K^+ channels are architecturally similar to Kv subtypes and present an extra transmembrane segment near the amino terminus, while the twin-pore K^+ (K_{2P}) channels are weak inward rectifier with four putative transmembrane domains and two pore domains.[2]

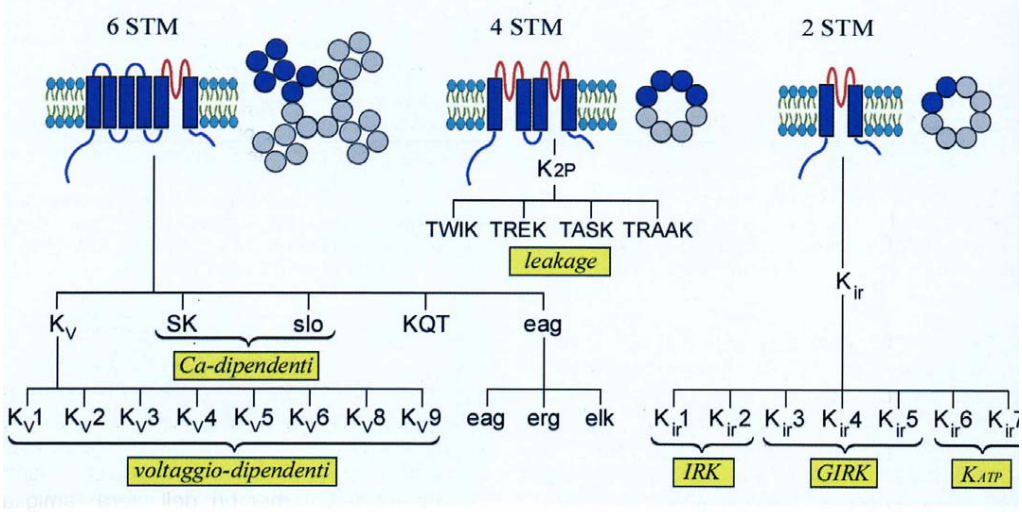

Figure 1.1 General architecture of K^+ channel families.

1.1.1 ATP-sensitive potassium channels

ATP-sensitive potassium (K_{ATP}) channels, discovered in the early 1980s in cardiac muscle and pancreatic β-cells, [3-6] are a weakly inward-rectifying K^+ channels that are inhibited by the non-hydrolytic binding of ATP, but activated by interactions with Mg^{2+}-bound nucleotides (Mg-nucleotides) at separate sites. K_{ATP} channels are postulated to act as sensors of intracellular metabolism, tuning the potassium permeability, and therefore the electrical activity, of a cell to its energetic balance[7]. The inhibitory effect dominates, and channels are closed, when cellular phosphorylation potential is high, but as metabolism decreases, the activating effect wins out and channels open. This metabolic sensing role is involved in many physiological processes, for example, it plays a role in the cascade linking insulin secretion to glycaemia in the pancreatic β-cell and participates in ischaemic pre-conditioning and thereby cardio-protection during heart failure.[6,8,9]

1.1.2 Protein architecture of K_{ATP} channels

K_{ATP} channels are formed by the combination of two types of proteins:[10] one, Kir6, is a ~50 kDa protein belonging to the inwardly rectifying potassium (Kir) channel family, the other, SUR (for sulphonylurea receptor), is a ~140-180 kDa member of the ATP-binding cassette (ABC) transporter family. The channel is a functional octamer of four Kir6 subunits assemble to form the pore, and each subunit is associated to four SUR subunits generating the regulatory site.[11,12]

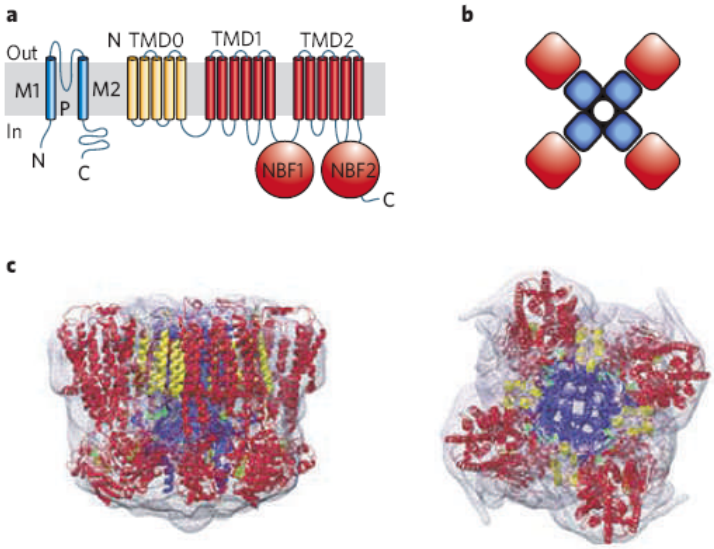

Figure 1.2 Protein architecture of K_{ATP} channels. **a**) Inward rectifier K^+ channel Kir6 subunits generate the channel pore and sulphonylurea receptor (SUR) subunits generate the regulatory subunit. TMD, transmembrane domain; NBF, nucleotide-binding fold; M1, M2, transmembrane helices; P, pore. **b**) The channel is a functional octamer of four Kir6 subunits, and each subunit is associated with four SUR subunits. **c**) Images at 18 Å resolution of the entire K_{ATP} complex viewed in the plane of the membrane (left) or from above the membrane (right).

Kir6 consisting of two transmembrane helices (M1 and M2) bridged by an extracellular loop that generates the narrow portion of the pore (H5) and control ion selectivity. Two members of the Kir6 family are known, Kir6.1 and Kir6.2 encoded by *KNCJ8* and *KNCJ11* genes respectively, which have about 70% aminoacid identity. SUR has strong homologies with other members of subfamily C of ABC proteins and are encoded by two genes, SUR1 and SUR2 with 70% homology. The SUR2 gene has two major splice variants, SUR2A and SUR2B, which differ by 42 aminoacids in the C-terminus. SURs possess the core domain composed of two transmembrane domains TMD1 and TMD2, with six transmembrane regions, and two cytoplasmic nucleotide binding folds NBF1 and NBF2.[12] In addition, it has a supplementary N-terminal domain TMD0 composed of five transmembrane helices which is linked to the core ABC domain by a cytosolic loop L0. Both Kir6, in the C-terminus, and SUR, in the sixth intracellular loop, have endoplasmatic reticulum (ER) retention sequences which prevent trafficking to the cell surface in the absence of the other subunit[13]. There is evidence[14] that TMD0 is crucial for trafficking Kir6.2 subunits to the surface membrane, and its role in controlling the gating of Kir6.2[15,16] suggests an intimate relationship with the poreforming subunit. In recombinant cells, truncation of the C-terminus of Kir6.2 (Kir6.2ΔC) removes its retention sequence (Arg-Lys-Arg), allowing it to form functional channel without SUR subunits[17] and indicating that channel inhibition by ATP is a property of Kir6 subunit. However, the ATP-sensitivity of Kir6.2ΔC is 10-20 fold lower than that of Kir6.2/SUR1 channels.

1.1.3 Nucleotide gating K_{ATP} channels

Several ligands affect K_{ATP} channel activity. These channels are inhibited by ATP (with or without Mg^{2+}) and activated by phosphatidylinositol-4,5-bisphosphate (PIP_2) by a direct interaction with Kir6.2 subunits of the channels. Sulphonylureas inhibit, and potassium channel-opener drugs activate, by interaction with the SUR subunit. In addition, in the presence of Mg^{2+}, ATP and ADP can activate the channel through interaction with the NBFs of SUR. Inhibition by ATP binding to Kir6.2 and activation by Mg-nucleotides is almost certainly the primary physiological regulatory mechanism. The metabolically controlled gate of the channel is located at the cytoplasmic end of the inner cavity. PIP_2 interaction provides an energetic pull to open channels, and ATP binding provides the energetic push to close the ligand-operated gate, perhaps through the physical link provided by a "slide helix", that lies along the plane of the membrane and physically connects the N terminus, which in Kir6.2 forms part of the ATP-binding site, to the cytoplasmic end of M1. MgATP binds to each of the ATP-binding sites (ABSs) that are formed at the interface between nucleotide-binding domains NBF1 and NBF2 in each sulphonylurea receptor subunit. ATP hydrolysis at the second site results in a conformational "activated" state that is transduced to an "override" of ATP inhibition at the

Kir6.2 subunit. The "activated" state persists through MgADP dissociation, and can be maintained by MgADP rebinding[7].

1.1.4 K_{ATP} channels diversity

K_{ATP} channels were first described by Noma[3] in cardiac ventricular myocytes, but they are also expressed in several tissue types including kidney, brain, skeletal muscle, heart, pancreatic β-cells and smooth muscle[18-21] and have been clearly identified in both sarcolemmal and mitochondrial membranes (sarc- and mito-K_{ATP}, respectively). Their functional role in many tissues occurs in response to metabolic changes: in the pancreas K_{ATP} channels underlie insulin secretion in response to an increase blood glucose concentration; in the heart they have a protective function in response to hypoxia or ischaemia. In the brain K_{ATP} channels have a similar protective function and are also involved in sensing the level of blood glucose. Opening of smooth muscle K_{ATP} channels causes relaxation, and in particular activation of vascular K_{ATP} channels suggest a role in contributing to the increase of blood flow in response to increased tissue metabolic demand. In skeletal muscle, K_{ATP} channels may play roles both in fatigue and in glucose uptake.[22]

K_{ATP} channels in different tissues are formed by different combination of Kir and SUR subunits, accounting for the tissue-specific properties of the channels. For example, the pancreatic β-cells are made up of SUR1/Kir6.2[23,24]; while SUR2A is associated with Kir6.2 in the cardiac sarcolemmal channel[25,26]; Kir6.1 and SUR2B probably constitute the major K_{ATP} channel in the vascular smooth muscle[27], while the channels in non-vascular smooth muscle are formed by SUR2B and Kir6.1 or Kir6.2. Different SUR subtypes respond differently to intracellular nucleotides, sulfonylureas, and potassium channel openers (KCOs), while Kir6.1 and Kir6.2 have similar ATP sensitivities. Therefore, the metabolic sensitivities and the pharmacological properties of a K_{ATP} channel are largely determined by its constituent SUR.

1.1.5 Potential therapeutic

The efflux of K^+ ions following opening of potassium channels is a mechanism for recovering (repolarization), maintaining (clamping) and/or enhancing (hyperpolarization) the resting state of the cell. Thus, the opening of potassium channels is a fundamental physiological means for reversal or prevention of depolarizing activity of the membrane and the role of these ion channels in cellular control and their opening by endogenous mediators, such as neurotrasmitters and hormones, is recognized as an inhibitory mechanism.

Potassium channels are a diverse and ubiquitous group of ion channel exhibiting a wide range of physiological roles in cell control processes, so drugs that decrease cell excitability by opening these channels could have a broad clinical potential.

In particular, considering the unique role that K_{ATP} play in the maintenance of the cellular homeostasis, potassium channel openers (KCOs) add to existent pharmacotherapy with potential in promoting cellular protection under conditions of metabolic stress, for example, preclinical evidences[28-35] have shown their potential therapeutic in asthma, urinary incontinence, hypertension, cardiac ischaemia, CNS desease.
The therapeutic usefulness of KCOs ultimately depends on their tissue selectivity which was majorly not achieved with first generation compounds. Thus, only four KCOs were introduced to clinical practice[32] including nicorandil (angina, hypertension), diazoxide (refractory hypertension), minoxidil sulfate (alopecia, refractory hypertension), and pinacidil (hypertension).

1.1.6 Asthma

Therapeutic potential of KCOs in lung disease primarly refers to asthma and chronic obstructive pulmonary disease (COPD)[36-41]. Bronchial asthma is characterized by wide variations over short periods of time in the resistance to flow in intrapulmonary airways. Accordingly, this disease is commonly treated with bronchodilators. Nowadays asthma is no longer viewed simply as a reversible airways obstruction, but is considered as an inflammatory illness resulting in bronchial hyperreactivity and bronchospasm. Thus, pharmacological treatment of asthma deserves anti-inflammatory and bronchodilator drugs. Recent trends in the development of new anti-asthmatic include PDE inhibitors, thromboxane A2 antagonists as well as KCOs[40].
Evidence for a bronchodilator potential KCOs dates back to 1986, when Allen *et al.*[42] showed cromakalim to reduce spontaneous, and to a lesser extent, cholinergic and histaminergic tone in preparations of guinea-pig trachealis. Effective relaxion of tone by KCOs was also shown in bovine[43] and in human airways[44]. KCOs exhibit stronger spasmolytic rather than anti-spasmogenic activity which resembles other classes of smooth muscle relaxants[36]. Corresponding to their spasmolytic efficacy *in vitro*, KCOs relax established bronchocostriction *in vivo*. However, in contrast to *in vitro* findings these drugs exhibit pronounced protective effects *in vivo* also when given prior to challenge. *In vivo* studies preferentially addressed the anti-spasmogenic activity of KCOs. Orally administration inhibited dyspnoea evoked by histamine in conscious guinea-pigs[45] while intravenously or intraduodenal administration inhibits bronchocostriction elicited by histamine, serotonin and bombesin.[45-49]
Bronchoconstriction often involves a reflex parasympathetic component, thus, neural effects of KCOs might be relevant for their therapeutic utility in asthma. Several studies show KCOs to potently inhibit the neurotransmitter release from cholinergic and non-adrenergic, non-cholinergic (NANC) neurons. KCOs more effectively inhibit Ach- or NANC-mediated bronchoconstriction because of stimulated neurotransmitter release as compared to bronchoconstriction because

of an exogenous supply of Ach or NANC neurotransmitters such as substance P or neurokinin A.[50,51] Inhibitory effects are probably mediated by hyperpolarizaion of sensory nerve endings via activation of prejunctional K^+-channels[32]. Beyond their effects on bronchoconstriction NANC neurotransmitters stimulate mucus secretion and the leakage of plasma from post-capillary venules, both of which are important in the pathology of asthma. These inflammatory effects of NANC neurotransmitters seem to be primarly due to the action of substance P.[52]
Airways hyper-responsiveness (AHR) to a plethora of physiological and pharmacological stimuli is a commonly observed characteristic of asthmatic patients. KCOs acutely reverse AHR by a direct effect on airways smooth muscle and they are able to prevent the development of AHR.
Molecular mechanism underlying the prevention of AHR by KCOs remain to be clarified.

1.1.7 Urinary incontinence

Urinary incontinence is classified in four major types: 1) stress incontinence; 2) urge incontinence; 3) reflex incontinence; 4) overflow incontinence (Table 1.1).

Table 1. 1 Subtypes of urinary incontinence[53]

Stress incontinence	Involuntary loss of urine during coughing, sneezing or physical activities; it occurs when intravescical pressure is higher than maximum urethral pressure but in the absence of detrusor activity
Urge incontinence	Characterized by abnormal spontaneous detrusor contraction unrelated to bladder volume; broadest category of urinary incontinence
Reflex incontinence	Involuntary loss of urine because of abnormal reflex activity in the spinal corde
Overflow incontinence	Involuntary loss of urine when the intravescical pressure exceeds the maximum urethral pressure because of an elevation of intravescical pressure associated with bladder distention but in the absence of detrusor activity

The etiology is multivariant and includes neurogenic and myogenic instabilities. Most incontinence is because of failure to store urine (storage dysfunction), only overflow incontinence is because of failure to empty urine (emptying dysfunction).
Pharmacological treatment of storage dysfunction should be aimed at decreasing detrusor overactivity, increasing bladder capacity, and/or increasing outlet resistance.

Pharmacological treatment of emptying dysfunction should be aimed at increasing detrusor contractility and/or decreasing outlet resistance[53-57]. Most widely used in pharmacological treatment are anti-muscarinic agents, which effectively inhibit bladder hyper-reflexia, but suffer from main side-effects, such as the inhibition of normal bladder smooth muscle contractility and the blockade of muscarinic receptors in other organs.
As an alternative to anti-muscarinics, KCOs should be able to decrease the bladder overactivity. Several KCOs were shown *in vitro* to relax the bladder smooth muscle in various species including man[58-61].

1.1.8 Nervous System

1.1.8.1 Peripheral Nervous System

The KCOs interfere with neurotransmission in peripheral parasympathetic neurones in the airways and the gastrointestinal tract[62]. This has led to suggestions of a presynaptic site of action, whereby the KCOs control the release of neurotransmitter. In contrast, cromakalim, nicorandil, and pinacidil failed to exert an inhibitory effect on noradrenaline release in rat isolated mesenteric artery[63]. The presynaptic inhibitory role of KCOs, therefore, appears to be selective for parasympathetic (cholinergic) innervation. Interestingly, cromakalim and pinacidil inhibited nicotinic acetylcholine receptor-mediated and voltage-dependent catecholamine secretion from bovine adrenal chromaffin cells[64]. Thus, K_{ATP} channels could be involved in regulation of catecholamine secretion mainly indirectly through voltage operated channels (VOCs).

1.1.8.2 Central Nervous System

Potassium channels play a pivotal role in the control of neuronal excitability, action potential, and neurotransmitter release within the CNS[65,66]. Activation of a variety of receptors (e.g., opioid, 5-HT_3, somatostatin, α_2-adrenoceptors) by the appropriate neurotransmitter alters the flux of K^+ ions from neurons[67]. Because of this role in normal CNS physiology, derangements in the function of K^+ channels may underlie several CNS diseases. Localization in the cerebral cortex and hippocampus would suggest that this channel may have a role in the processing of memory. Studies *in vitro* and in animal models indicate the potential clinical utility of KCOs for diseases of the CNS.
The genesis and propagation of non physiologic electrical impulses are the hallmark of epilepsy. Thus, the hyperpolarization (and restraining) of excitable cells through the opening of K^+ channels could demonstrate therapeutic benefit in this setting. Opioids exert their analgesic effects by binding to opiate receptors, which leads to opening of K^+ channels and neuronal hyperpolarization[67]. Morphine induced antinociception in mice tail-flick tests is mediated by the opening of K_{ATP} channels[68]. These observations would suggest a potential role for KCOs as analgesics. During anoxic conditions, neuronal

depolarization is due, at least, to the release of large concentrations of excitatory amino acids, such as glutamate, which may be involved in long-term ischaemia-induced damage to the brain[69]. In *in vitro* experiments, diazoxide and somatostatin were shown to prevent anoxia-induced depolarization of CA3 hippocampal neurons following the opening of K^+ channels[70]; these effects were inhibited by pretreatment with glyburide. The authors proposed that KCOs may prevent anoxia-induced damage to hippocampal neurons by inhibiting the release of excitatory amino acids. Schmid-Antomarchi *et al.* [71] noted that the order of potency of the KCOs (levcromakalim > nicorandil > cromakalim > diazoxide > pinacidil) was found to be different from that in either the pancreatic β-cell or in smooth muscle, possibly indicating a difference between the target K^+ channel in this brain region and that in other tissues. The K_{ATP} channel in neuronal tissue is not the classical (Type 1) channel found in pancreatic β -cells or heart, but a large-conductance non-rectifying version (Type 2) [72].

1.1.9 Skeletal Muscle

In skeletal muscle, K_{ATP} channel activity has been shown to increase upon intracellular acidification[73]. Falls in intracellular pH reduce the inhibitory effect of ATP on K^+ channels in frog skeletal muscle. This could mean that during increased muscle exercise and consequent lowering of pH, K_{ATP} channel-induced hyperpolarization could compensate for a decrease in electrical excitability and prevent spontaneous contractions from occurring. KCOs, however, increased open probability of an ATP-sensitive and an ATP-insensitive K^+ channel in human skeletal muscle[74]; the effect of the KCOs on both channels were blocked by glibenclamide. Ischaemia-induced damage in a rat skeletal muscle, like cardiac and neuronal ischaemia, has been shown to be prevented by cromakalim[75]. This result, and the observation that cromakalim restores the membrane potential of depolarized human skeletal muscle fibres[76], indicate that KCOs may be useful for the treatment of peripheral vascular disease. In rat skeletal muscle, Angersbach and Nicholson[77] demonstrated that KCOs, but not Ca^{2+} antagonists or hydralazine, selectively increase blood flow to collateral vessels in a previously ischemic limb. Weselcouch *et al.* [78], however, suggested that KCOs would not be beneficial in treatment of skeletal muscle ischaemia *in vivo,* but may be useful in preserving skeletal muscle function in cases of ischaemia followed by reperfusion. Studies in human muscle have implicated SK_{Ca}, (apaminsensitive) channels in the condition myotonic muscular dystrophy, characterised by muscle stiffness[79]. Although K^+ channel subtypes other than K_{ATP} channels exist in skeletal muscle (SK_{Ca}, BK_{Ca}, delayed rectifier K^+ channel)[80], however, selective openers are awaited to determine their role and the therapeutic potential of activation.

1.1.10 Myocardial Ischaemia

A beneficial role of KCOs in myocardial ischaemia[81-84] was first suggested by investigations on nicorandil[85]. Later, Grover *et al.* [86] studied the anti-ischaemic effects of KCOs in a model of ischaemia and reperfusion in which complicating vasodilator effects were avoided and thus direct cardioprotective activity could be verified. These authors showed pinacidil and cromakalim to protect isolated rat heart subjected to 25 min global ischaemia followed by 30 min reperfusion; concentrations needed to increase the time to ischaemic contracture by 25% were used as indicators of anti-ischaemic potency. Cardioprotective properties of other KCOs including bimakalim and aprikalim could be shown in similar models[87-92]. Putative involvement of K_{ATP} channels with the mechanism of myocardial preconditioning further increased the interest in these channels.

KCOs mimic preconditioning in the absence of ischaemia, whereas K_{ATP} blockers such as glibenclamide[93] and 5-hydroxydecanoate (5-HD)[94] were shown to antagonize preconditioning. The original hypothesis to explain these observations involved sarcolemmal K_{ATP} channels; shortening of action potential duration (APD) was viewed as a putative mechanism of the cardioprotection by KCOs. However, KCOs exert their cardioprotective effects without a significant cardiodepression indicating a lack of importance of sarcolemmal K_{ATP} channels to mediate cardioprotection. This view is supported by the abolition of cardioprotective effect of KCOs in the presence of 5-HD[94] that is a selective blocker of mitochondrial K_{ATP} channels[95], while the sarcolemmal K_{ATP} channels blocker HMR1883 failed to abolish preconditioning[96].

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2 MYOCARDIAL ISCHAEMIC PRECONDITIONING (IPC)

2.1 Ischaemia-Reperfusion (I/R) Injury

I/R injury of the heart is characterized by reversible contractile dysfunction, known as stunning, and irreversible injury leading to cardiomyocytes death and myocardial infarction. Myocardial stunning, is defined by the mechanical dysfunction that persists after reperfusion, despite the absence of irreversible damage and despite restoration of normal coronary flow.[1]

Two major hypotheses have been proposed to explain the mechanism of myocardial stunning:[2] 1) the oxyradical hypothesis, in which it is caused by the generation of ROS; 2) the Ca^{2+} hypothesis, in which it is caused by a transient Ca^{2+} overload on reperfusion. The final lesion responsible for the contractile depression appears to be a decreased responsiveness of contractile filaments to Ca^{2+}. Recent evidence suggests that Ca^{2+} overload may activate calpains, Ca^{2+}-dependent neutral proteases, resulting in selective proteolysis of myofibrils. The time required for resynthesise damaged proteins would explain in part the delayed recovery of function in stunned myocardium. The ROS and Ca^{2+} hypotheses are not mutually exclusive and are likely to represent different component of the same pathophysiologic cascade. For example, increased ROS formation could cause cellular Ca^{2+} overload, which would damage the contractile apparatus of the cardiomyocytes. ROS generation could also directly alter contractile filaments in a manner that renders them less responsive to Ca^{2+} (e.g., oxidation of critical thiol groups). An important implication of the phenomenon of myocardial stunning is that so-called chronic hibernation may be the result of repetitive episodes of stunning, which have a cumulative effect and cause protracted post-ischaemic dysfunction. Besides direct effects of ROS and Ca^{2+} on Ca^{2+}-regulatory proteins and contractile machinery, ROS and intracellular Ca^{2+} overload modulate cardiomyocyte contractility through phosphorylation of contractile proteins. A variety of protein kinases are activated in response to oxidative stress, and this process is pivotal in regulating the contractility. Particularly, PKC and p38 mitogen-activated protein kinase (MAPK) have been implicated in the pathogenesis of ischemic contractile dysfunction.[3,4]

Paradoxically, reperfusion can exacerbate the damage occurring during the ischaemic period. This is known as reperfusion injury and is accompanied by enzyme release and morphological changes characteristic of necrosis.[5-7]

In addition to the necrotic cell death that represents the major damage to the reperfused heart there is also evidence that some myocytes around the periphery of the infarct die by apoptosis.[8,9] The myocardial cell appears to be primed by a sequence of events during the ischaemic period that “sets up” the cell for an irreversible injury, which is then triggered by reperfusion.

During ischaemia the intracellular acidosis that results from anaerobic glycolysis drives a series of coupled exchanges in which the Na^+/H^+ exchanger removes H^+ and accumulates Na^+ in the cell, which is then coupled to Ca^{2+}-influx by reverse-mode Na^+/Ca^{2+} exchange activity, resulting in a Ca^{2+} accumulation. Moreover, during ischaemia, the intracellular ATP concentration falls with consequent reduction in activity of the Na^+/K^+ ATPase and increase in $[Na^+]i$. Additional effect of ischaemia includes inihibition of the sarcoplasmic reticulum Ca^{2+}-release channel (RyR). At the moment of reperfusion, the cell is characterized by high levels of intracellular Na^+, Ca^{2+} and possibly mitochondrial reducing power (NADH, $FADH_2$) and the sarcoplasmic reticulum is loaded with Ca^{2+}. Reperfusion triggers an injury cascade that combines reactivation of electron transport and oxidative phosphorilation, which increases the availability of ATP, with the existing Ca^{2+} load to produce a strong hypercontracture, which in the intact tissue leads to mechanical damage. There is also a secondary Ca^{2+} influx in the Na^+/Ca^{2+} exchanger driven by a reperfusion induced influx of Na^+ via the Na^+/H^+ exchanger stimulated by the washout of extracellular acidosis. In addition to these metabolic effects, re-energization of the mitochondria products a burst of reactive oxygen species and rapid mitochondrial repolarization which leads to Ca^{2+} accumulation and opening of mitochondrial permeability transition pores (MPTP) and thus the initiation of cell death at reperfusion.

Understanding the causes of reperfusion injury and devising ways of preventing it is of major clinical importance in cardiac surgery and the treatment of coronary thrombosis. There is increasing evidence that mitochondrial dysfunction plays a central role in mediating both the necrotic and apoptotic components of reperfusion injury, and that one of the most effective ways of protecting hearts from such injury is to subject them to brief ischaemic periods with intervening recovery periods before the prolonged period of ischaemia is initiated. This phenomenon is known as ischaemic preconditioning (IPC).[5,7,10-13]

Some authors have also hypothesized that functional recovery after ischaemia is predicated on the ability of the myocyte to maintain sufficient substrate metabolism during ischaemia. This hypothesis starts from the observation that glucose metabolism via the polyol pathway, and consequently aldose reductase (AR) activity, is increased in ischemic hearts[14-17] and recently, it was demonstrated that inhibition of AR protects rat hearts from ischemic injury and improves functional recovery upon reperfusion.

During ischaemia, lack of oxygen leads to rapid stimulation of glucose uptake, glycogenolysis and glycolytic flux.[18,19] Moderate reduction in blood flow and oxygen supply result in increased glucose extraction and glycolysis with no changes in glucose uptake, whereas severe reductions result in increased myocardial glucose uptake, glucose extraction and significantly increased glycolysis. The induction of ischaemia is followed by recruitment of the glucose transporters GLUT-4 and GLUT-1 from the intracellular stores to the

plasma membrane.[20-22] Stimulation of glucose transport during ischaemia is reflected by the increased glycolytic flux.[18-22] However, prolonged ischaemia results in inhibition of glycolysis due to the increased levels of lactate, protons and decreased availability of NAD^+.[16,17,19] Recent studies have shown that ischaemia increases aldose reductase activity and NAD^+ use via this pathway. As a result glycolysis is impaired due to shortage of NAD^+ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[14,16,17]

Sustaining glycolysis during ischaemia is an important source of ATP for maintaining the activities of sarcolemmal Na^+/K^+ATPase and the sarcoendoplasmic Ca^{2+}-ATPase.[23-25] There is evidence to suggest that glycolysis preferentially supplies ATP for these critical ion pumps[24,25] and thus, even if glycolytic metabolism does not provide sufficient energy for to provide enough ATP to maintain cardiac contraction, it may provide adequate energy to maintain the ionic homeostasis and thus preserve viability during the ischaemic insult.

Fatty acids have been shown to be detrimental to ischaemic myocardium. Accumulation of long-chain fatty acids may adversely affect intermediary metabolism by increasing $NADH/NAD^+$ ratio; increasing acetyl-CoA/CoASH with consequent increases in citrate levels and inhibition of PFK; inhibiting pyruvate carboxylase and increasing the amounts of long chain acyl carnitine (LCA).

Numerous experimental studies have convincingly demonstrated that maintenance of glycolytic metabolism by the ischaemic myocardium can preserve viability,[16,17,26-28] can delay ischaemic contracture and can prevent reversible cell damage.[29] Glycolysis is required to support membrane function and in particular, maintain sodium and calcium homeostasis.[23-25,30] It has been shown repetely in numerous studies that protection myocardium during ischaemia by favourably modifying metabolism reduces ischaemic injury and improves functional recovery upon reperfusion.[17,28,31-34] Thus, there is excellent experimental and clinical data to support the concept that increased glycolytic flux, induced by increased exogenous glucose and reduced serum fatty acids, assists in protecting the ischaemic myocardium and improving survival and function after acute ischaemic episodes. Moreover, recent studies have shown that targeting aldose reductase may provide a novel approach to protecting ischaemic myocardium.[35]The reduction in ischemic injury due to aldose reductase inhibition was associated with attenuation of the rise in cytosolic redox ($NADH/NAD^+$ ratio) and improved glycolysis in ischemic myocardium.[16,34,36]

Furthermore, AR inhibition improved myocardial glucose oxidation upon reperfusion[34]and beneficial changes in ATP and ion homeostasis.[16,34]

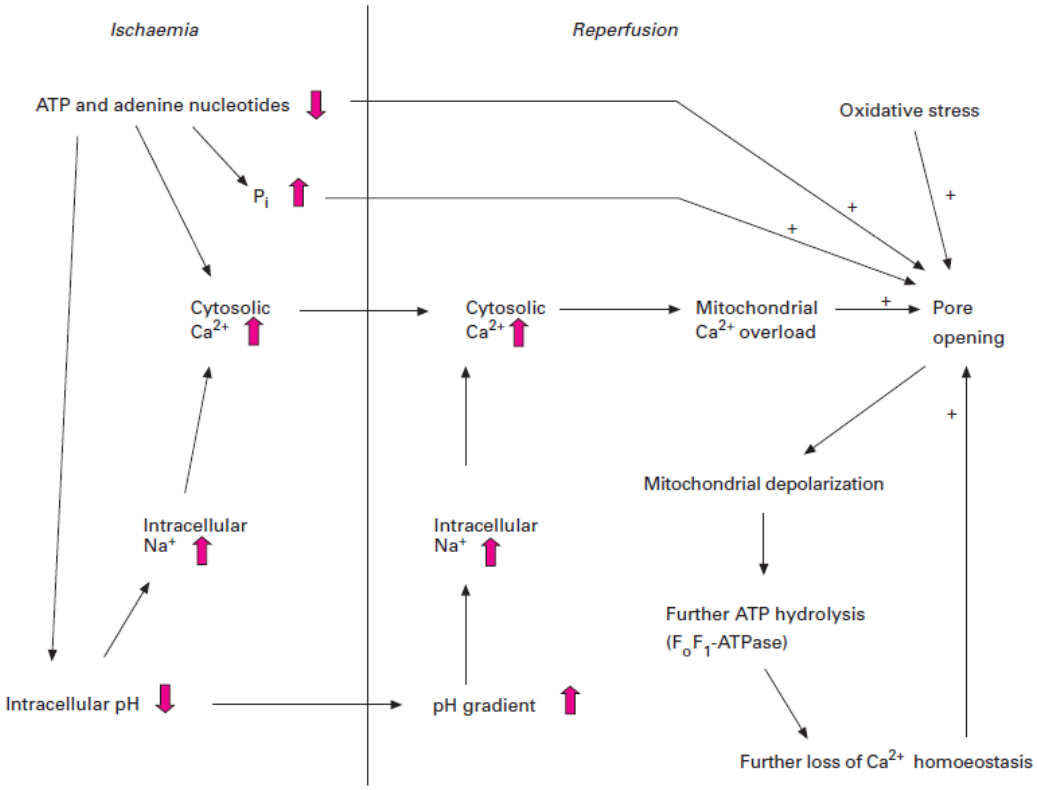

Figure 2.1 Involvement of MPTP in I/R induced cell death. ATP dissipation during ischaemia leads to rises in resting cytosolic free [Ca^{2+}] and P_i. Reperfusion leads to excessive mitochondrial Ca^{2+} uptake. Mitochondrial Ca^{2+} overload together with oxidative stress and the prevailing high P_i and low ATP provoke MPTP opening.

2.2 Preconditioning

Ischaemic Preconditioning (IPC) has been exploited as a powerful endogenous form of cardioprotection. IPC was first discovered by Murry[37] and associates, who demonstrated that a brief period of repetitive cardiac I/R exerts a protective effect against subsequent lethal periods of ischaemia, resulting in a marked reduction in infarct size, the extent of stunning and the incidence of cardiac arrhythmias.[38,39] This "self-defence" mechanism also induces an improvement of the post-ischaemic recovery[40] and a protection of the coronary endothelium.[41]

The development of IPC shows two distinct phases: an early phase (or classic IPC) that lasts for 1 to 3 hours following the preconditioning stimulus and a delayed one (or second window of IPC) that lasts for 24 to 96 hours. The initial early IPC does not appear to depend on new protein synthesis because of the rapid onset and because the inhibition of protein synthesis does not block this phase, while the second window of IPC involves up-regulation of genes rather than post-traslactional modification of proteins.[42-44]

The exact mechanisms involved in preconditioning are still being debated, but several processes have been implicated. In the longer-term effects, probably caused by stimulation of the transcription of specific genes, perhaps through a mechanism activated by free radicals and stress-activated protein kinases, of particular interest may be the up-regulation of heat shock proteins since recent data have shown that heat shock specifically up-regulates the expression of liver mitochondrial Hsp25 and this is associated with desensitisation of mitochondrial permeability transition pore (MPTP) opening to Ca^{2+} and $HgCl_2$ in isolated mitochondria.[45] It is also known that heart mitochondria from mice in which Hsp25 is down-regulated are more sensitive to MPTP opening and also exhibit hallmarks of oxidative stress including oxidatively damaged adenine-nucleotide traslocase (ANT).[46] The mechanisms responsible for the short-term effects of preconditioning include the activation of protein kinase C (PKC). This may be mediated either by reactive oxygen species (ROS) released during the short intervening reperfusion periods, or by factors released during the brief ischaemic periods such as adenosine, bradykinin, noradrenaline, and opioids. Thus, PKC inhibitors and free radical scavengers antagonise IPC, whilst adenosine agonists and PKC activators mimic the effect.[47-49] The ultimate target of these kinases is unknown, although it may be significant that activation of PKCε and its translocation to mitochondria has been reported to be important for preconditioning.[50,51] There is also evidence for an involvement of sulphonylurea-sensitive K_{ATP} channels, since K_{ATP} channel openers such as diazoxide can mimic IPC whilst blockers such as glibencamide inhibit.[52,53] Furthermore, PKC-dependent activation of plasma membrane K_{ATP} channels by IPC has been demonstrated.[54] The mechanism of protection from cardiomyocyte apoptosis by IPC appears to be due to protection of mitochondria. It has been shown that IPC reverses many aspects of mitochondrial dysfunction induced by I/R, including loss in the activity of the redox-sensitive Krebs cycle enzyme α-ketoglutarate dehydrogenase, declines in NADH-linked ADP-dependent mitochondrial respiration, insertion of the pro-apoptotic Bcl-2 protein Bax into the mitochondrial membrane, and release of cytochrome ***c*** into the cytosol.[55]

This protection of mitochondria by IPC is mediated by activation of survival signaling that converges on prevention of the MPTP opening.[56-58]

The survival signalling includes the PKCε–mitochondrial K_{ATP} (mito-K_{ATP}) channel pathway,[59] the phosphatidylinositol 3-kinase (PI3K)/ Akt pathway,[60] the Janus kinase (JAK)/signal transducers, and activators of transcription (STAT) pathway.[61]

2.2.1 MPTP and its role in cell death

Mitochondria play critical roles in both the life and death of cells. In healthy cardiac myocytes, their primary function is the provision of ATP through oxidative phosphorylation to meet the high energy demands of the beating

heart. Glycolysis alone is unable to meet these demands even in the resting state and inhibition of oxidative phosphorylation, as occurs in anoxia or ischaemia, leads to impairment or cessation of normal heart function. However, latent within mitochondria, there are mechanisms that, once activated, convert the mitochondria from organelles that support the life of the cell to those that actively induce both apoptotic and necrotic cell death. The switch in roles is mediated by the opening of a nonspecific pore in the mitochondrial inner membrane, known as the mitochondrial permeability transition pore (MPTP). This normally remains closed, but can open under conditions of cellular stress with dire consequences.

The release of proapoptotic molecules, such as cytochrome *c* or apoptosis-inducing factor, from mitochondria triggers the activation of caspases that finally leads to cellular destruction.[62] This process is associated with opening of the MPTP, a ‘‘megachannel’’ spanning both the inner and the outer mitochondrial membrane. It can exist in various subconductance states. In the fully opened state, it allows molecules up to a molecular weight of 1.5 kDa to pass.[63] Accordingly, opening of the MPTP leads to a breakdown of the mitochondrial membrane potential, causing failure of oxidative phosphorylation and ATP depletion.[64] The pore is formed from a complex of the voltage dependent anion channel (VDAC), the adenine-nucleotide traslocase (ANT) and cyclophilin-D (CyP-D) at contact sites between the mitochondrial outer and inner membrane. Ca^{2+} is the major physiological activator of the MPTP, other triggers are ROS and nitrogen species, mitochondrial depolarization and high levels of inorganic phosphate (P_i). In contrast, hyperpolarization of the mitochondrial membrane as well as high levels of ATP and adenosine diphosphate (ADP) are known to inhibit the pore.[63]

The MPTP opens under condition of high matrix Ca^{2+}, ROS, high NADH, depletion of adenine nucleotides and loss of membrane potential ($\Delta\psi$),[65-67] conditions that occur during ischaemia-reperfusion.[68,69]

Low pH, as occurs during ischaemia, inhibits MPTP, but intracellular pH is rapidly restored on reperfusion, thus allowing activation of the pore. Furthermore, the reduced $\Delta\psi$ during ischaemia would limit uptake of mitochondrial Ca^{2+}, but the reintroduction of oxygen on reperfusion would reconstitute $\Delta\psi$ (stimulating Ca^{2+} uptake into the mitochondria) and the introduction of oxygen will also allow generation of ROS. Thus the conditions that exist right at the start of reperfusion are ideal to stimulate opening of the MPTP.

Recent data shown that mito-K_{ATP} channels interact with MPTP. In particular, it has been shown that activation of the mito-K_{ATP} channel leads to a depolarization of mitochondria by increasing K^+ influx, thus reducing the Ca^{2+} influx into mitochondria.[70] A reduced intramitochondrial Ca^{2+} concentration is known to prevent opening of the MPTP. Accordingly, inhibition of mito-K_{ATP} channel might hyperpolarize mitochondria by decreasing K^+ input, thus

increasing the Ca^{2+} influx and facilitating MPTP opening. On the other hand, hyperpolarization of mitochondria is commonly considered to be a MPTP-inhibiting factor.[71] A fall in mitochondrial potassium concentration due to inhibition of the mito-K_{ATP} channel should alkalize the mitochondria matrix because of a reduced activity of the K^+/H^+exchanger.
A rise in pH is known to increase the open probability of the MPTP.[72]. Furthermore, an alkaline pH elevates the activity of the P_i/OH^- antiporter of the inner mitochondrial membrane and, consequently, matrix phosphate concentrations. Phosphate is a physiological modulator of the MPTP as high phosphate increases the open probability. A different explanation of the interaction between the mito-K_{ATP} channel and the MPTP has been suggested by Costa *et al.*,[73] who reported that inhibition of the MPTP by mito-K_{ATP} channel opening can be abolished by inhibitors of protein kinase C (PKCε) as well as by a scavenger of reactive oxygen species. They proposed that mito-K_{ATP} channel activation increases production of H_2O_2, which in turn blocks the MPTP by activating PKCε. The mito-K_{ATP} channel is able to modulate the opening probability of the MPTP[74] that is related to both apoptotic and necrotic cell death (e.g., in ischaemia), so modulation of this mitochondrial ‘‘megachannel’’ by controlling the state of the mito-K_{ATP} channel may offer therapeutic benefit for various diseases.

2.2.2 Signal trusduction in IPC

IPC involves variuos signaling as triggers and/or end effectors and an important role is undoubtely played by the activation of cardiac K_{ATP}.
The triggers of IPC can be classified as receptor-dependent and receptor-independent: the former are activators that elicit their action by specific receptor interaction, such as adenosine, bradykinin and opioids which bind to G protein-coupled receptors (GPCRs); the latter are endogenous substances that do not need a selective binding with the receptor such as nitric oxide (NO), free radicals and Ca^{2+}.

Receptor-dependent Triggers
GPCRs are coupled with $G_{i/o}$, wich consists of heterotrimeric G protein $G_{\alpha\beta\gamma}$, or with a G_q family, which consists of the heterotrimeric G protein, $G_{\alpha q}$. Activation of $G_{i/o}$-coupled receptors induces dissociation of G_α from $G_{\beta\gamma}$ which activates phospholipase C (PLC) and a resultant generation of the second messengers D-myoinositol 1,4,5-triphosphate and diacylglycerol, leading to activation of PKC, an essential step for mediating IPC.[75] Conversely, signaling pathways linked with activation of $G_{\alpha q}$ also converge at the level of PLC.[76] General agreement exists that adenosine is a major GPCR in triggering IPC.[77] Cardiac adenosine levels are increased by brief periods of ischaemia in rats, dogs and pigs[78-82] and improve the oxygen supply-demand balance through the dilatation of resistance vessels and the reduction of the cardiac inotropism. These effects

are mediated by A_1 and A_3 receptors, while the A_2 receptor subtypes in the IPC seems to be excluded.[83,84]

Like other Gq-coupled receptors, the activation by autacoid in preconditioned tissue triggers a multiple kinase cascade through phosphorylation reactions, leading to the rapid post-translational modifications of proteins that regulate cellular resistance to I/R injury.[85,86] Among the autacoids, kinins have been implicated in myocardial IPC.[87] The most abundant of these, bradykinin, play an important role, during a short IPC in pigs[82] or a single cycle of ischaemia-reperfusion in rabbits, and it has been suggested that adenosine and bradykinin can act synergistically.[88]

In addition to GPCR coupled agonists, it was found that growth-factor receptor (GFR) agonists that are coupled with receptor tyrosine kinase (RTK) are equally effective in cardioprotection. The mechanism of GFR-mediated preconditioning effects is not well understood but may share common intracellular signaling pathways to GPCRs. These pathways ultimately converge on activation of end-effector systems because GFR activation is also known to be coupled with activation of PLC and resultant activation of PKC.[89] Moreover, recent studies suggest that GPCR activation induces transactivation of GFRs *via* a redox-sensitive mechanism.[90,91] Thus, it is conceivable that coordinated activation of GPCR and GFRs provokes efficient cardioprotective signal transduction in IPC.

Opioid receptors are expressed in rat cardiac myocytes and their activation is involved in the reduction of infarct size.[92,93] Several experimental data demonstrate that the δ opioid receptor type is involved in IPC.[94,95] In particular, the δ_1 subtype stimulation can induce a delayed cardioprotection[96], while a possible role of the κ opioid receptor in IPC is controversial.[97]

The JAK-STAT pathway is a stress-responsive mechanism that transduces signals from the cell surface to the nucleus, thereby modulating gene expression. Recent studies have demonstrated that myocardial I/R induces various members of the cytokine superfamily, such as interleukin (IL)-6[98] and tumor necrosis factor (TNF)-α, and induces rapid activation of the JAK-STAT pathway.[99] Although an immediate effect of JAK/STAT signaling during ischaemia and reperfusion may be detrimental to cardiomyocyte survival and cardiac function,[100] activation of this signaling pathway ultimately promotes cytoprotection.[101] Emerging evidence suggests that JAK/STAT signaling plays an important role in the development of the cardioprotective phenotype associated with IPC.

A potential role of other triggers, such as prostaglandins, noradrenaline, angiotensin and endothelin is still debated.[102-106]

Receptor-independent Triggers

Nitric oxide (NO), whose cardiac levels are increased during ischaemia, has been indicated as an important endogenous factor involved in cardioprotection.

Data from several laboratories have implicated activation of cyclic GMP dependent protein kinase (PKG), perhaps by nitric oxide, in the signalling pathway for IPC. Thus it has been reported that exogenous NO-donors and PKG activators can induce preconditioning whilst NO scavengers and PKG inhibitors prevent preconditioning.[107-109] Pharmacological studies from Garlid's laboratory have led them to conclude that PGK and PKCε work in concert to induce IPC through an effect on the mito-K_{ATP}. They propose that cGMP activates PKG localized at the cytosolic surface of the mitochondrial outer membrane and that this phosphorylates some target proteins which in turn can somehow activate PKCε residing in the intermembrane space of mitochondria. This PKCε then would phosphorylate the mito-K_{ATP} channel to mediate preconditioning.[108]

A growing body of evidence suggests that ROS play a crucial role in signal transduction mediated by IPC. Vanden Hoek and associates[48] first demonstrated the loss of preconditioning protection with antioxidants in cardiomyocytes.

Mediators and end-effectors in IPC

PKC has been indicated as one of the main intracellular mediators of IPC since inhibition of PKC has been shown to block the protection afforded by IPC and pharmacological activators of PKC are cardioprotective.[110] There remains some controversy over which of the many PKC isoforms may be involved in IPC, whether they translocate to the particulate fraction and how they exert their effects.[110,111] Nevertheless, there is a large body of evidence to implicate PKCε a novel PKC isoform that has consistently been implicated in the cardioprotective signal transduction,[112,113] as an important player in IPC.[110] Thus hearts from PKCε knockout mice do not exhibit IPC[110,114] whereas transgenic mice with cardiac specific over-expression of PKCε or expression of an activator of PKCε are protected from reperfusion injury.[110,115-117]

Although a role for PKCε in IPC seems established, the mechanism(s) by which it exerts its effects are less clear, some studies have reported PKCε translocation to the particulate fraction, including mitochondria[110,118-120] and have suggested a direct inhibition of the MPTP by PKCε involving phosphorylation of components of the MPTP such as VDAC.[110,119-121]

It is also uncertain how ischaemic preconditioning activates PKCε, although several pathways may be involved. Factors released during the brief ischaemic periods such as adenosine, bradykinin, noradrenaline and opioids may bind to their G protein-coupled receptors to stimulate phospholipase C, producing

diacylglycerol that activates PKC. Indeed, all of these factors can pharmacologically precondition the heart.[110,112]

Accumulating evidence supports a role for the modest increase in ROS that occurs during IPC protocol in the activation of PKC.[122,123] Thus ROS are known to activate PKC in the isolated heart[123-125] and IPC can be prevented if free radical scavengers are present during the preconditioning phase.[110,123,126-128] Oxidation of critical cysteine residues on PKC isoforms is known to cause their activation[110,129,130] and thus provides a mechanism by which ROS could activate PKC.

The unique feature of IPC is the memory of cardioprotection, which lasts for up to 2 h after the discontinuation of the preconditioning stimulus.[131] The underlying mechanisms for memory of cardioprotection generated by IPC have been a subject of extensive research for many years. It has been suggested that mito-K_{ATP} channels and PKC create a self-perpetuating cycle during the memory of IPC.[132] Recent studies[133,134] raised the hypothesis that ROS generated through the activation of mito-K_{ATP} channels play a pivotal role in the memory of cardioprotection. In line with this hypothesis, Juhaszova and associates[134] suggested that the characteristic memory of IPC is mediated by moderate, reversible, and sustained mitochondrial swelling, which causes increased generation of ROS and consequent redox activation of PKC_{ε}, A growing body of evidence indicates that PKC_{ε} and PI3K play a crucial role in cardioprotective signal transduction mediated by IPC.[135] Important cross-talk between PKC_{ε} and PI3K exists in mediating the memory of cardioprotective signal transduction in IPC. It has been demonstrated that PI3K exists upstream of PKC in cardioprotection mediated by IPC in the isolated and perfused rat heart model.[136] However, more recent study[137] suggests that PKC_{ε} and PI3K exist in parallel positions, and the activities are regulated at least in part by each other's kinase.

Although the end- effector phenomena of IPC have not yet been completely identified, several observation report that in IPC there is a reduced accumulation of catabolites, such as lactate.[138] Furthermore, IPC reduces intracellular acidification, an indication of decreased anaerobic glycolysis. Other putative end-effector include: Na^+/H^+ axchanger, cytoskeleton changes down regulation of TNFα and the activation of cardiac, and in particular, mitochondrial K_{ATP} channels.[139,140]

2.2.3 Cardiac K_{ATP} channels

K_{ATP} channels exist in high density in the sarcolemmal membrane (sarc-K_{ATP}) as well as the mitochondrial membrane (mito-K_{ATP}) of cardiomyocytes. Both sarc- and mito-K_{ATP} channels in the cardiovascular system might have a physiological role in modulating cardiac function, particularly under conditions

of metabolic stress, such as hypoxia, ischaemia, and metabolic inhibition when intracellular ATP stores are reduced. Under normoxic conditions, the K_{ATP} channel exists mainly in a closed, inactive form. However during myocardial ischaemia, as the intracellular ATP concentration falls and ischemic metabolites (ADP, lactate, H^+) accumulate, the probability of the channel being open increases. This results in an enhanced outward repolarizing flow of K^+ and cell membrane hyperpolarization. Consequently, the myocardial action potential duration (APD) is shortened, the voltage-dependent calcium current and myocardial contractility are decreased thereby leading to ATP preservation during ischaemia. Thus, it is thought that K_{ATP} channels exert a protective property in myocardial ischemic diseases.[141]

Anti-ischaemic properties of cardiac K_{ATP}

It is well known that the plateau phase of the cardiac action potential (AP) shortens markedly and contractions decrease during metabolic depression induced by ischaemia, hypoxia, anoxia. However, the mechanisms responsible for these functional alterations and their significance to myocardial survival during ischaemia remain to be fully understood. The more rapid repolarization of the AP could be due to:

1) a decrease in inward current;
2) an increase in outward current, or
3) a combination of these changes.

Voltage-clamp studies imply that the primary alteration in membrane currents during metabolic poisoning, anoxia, and hypoxia is the development of a very large time-independent outward K^+ conductance,[142,143] resulting from the activation of K_{ATP}.[144-147]

Noma[148] postulated that K_{ATP} activation and a fall in AP duration might lead to a preservation of cellular ATP and prevention of irreversible ischemic injury.

The mechanism by which a K_{ATP}-induced decline in AP duration protects the myocardium during ischaemia remains to be defined, but two possible mechanisms have been suggested. The first is a marked reduction of the time for Ca^{2+} influx via voltage sensitive Ca^{2+} channels, and the second is an increased window of time during which the Na^+-Ca^{2+} exchanger may operate in forward mode activity to extrude Ca^{2+}. Decreased Ca^{2+} influx could directly improve the ability of cells to maintain appropriate $[Ca^{2+}]_i$ levels by reducing the load on the Ca^{2+} pump and the Na^+-Ca^{2+} exchanger at a time when both of these Ca^{2+} extrusion mechanisms are very likely depressed: the former because of reduced energy supply and the latter because of the marked depolarization that occurs during ischaemia as a result of the shift in the K^+ equilibrium potential (E_K).

The presence of a short AP also means that the cell will spend a greater period of time at membrane potentials negative to the reversal potential of the Na^+-Ca^{2+} exchanger and favourable for Ca^{2+} extrusion via forward mode activity. A

short AP and reduced Ca^{2+} influx may also indirectly reduce the consumption of high energy phosphate stores. This energy would be used in contractile activity and, to a lesser extent, for the maintenance of $[Ca^{2+}]_i$. Noma[148] originally postulated that since contraction would be indirectly depressed by a decline in AP duration, K_{ATP} activation would indirectly inhibit a major site of cellular energy consumption and reduce the rate of decline in $[ATP]_i$ during the no-flow period. It is clear that contractile failure during anoxia[146] and metabolic poisoning[147] is principally the result of activation of K_{ATP}, Ca^{2+} current, Ca^{2+} release from the sarcoplasmic reticulum.
The ability of K_{ATP} to alter AP duration may be the most important mechanism by which these channels contribute to myocardial preservation during ischaemia.[149]

2.2.4 Sarcolemmal K_{ATP} channels

It was initially believed that opening of sarcolemmal K_{ATP} channels (sarc-K_{ATP}) was protective because it shortened action potential duration (APD), thereby reducing Ca^{2+} entry to the cytosol.[149]
Sarcolemmal K_{ATP} in cardiac muscle are formed from Kir6.2 and SUR2A subunits and have a unitary conductance of 70-90pS. They open in the absence of ATP and are closed by micromolar concentrations of ATP (Ki ~23-30 μM). However, while free ATP^{4+} as well as MgATP are able to close the channel, maintenance of channel activity relies upon MgATP through channel phosphorylation, which may explain the transient nature of channel opening observed during prolonged metabolic inhibition. The ATP-sensitivity of the channel is decreased by nucleotide diphosphates (ADP, GDP, UDP) and acidosis, conditions likely to exist in the ischaemic myocardial cell. Sarc-K_{ATP} are also regulated by phosphorylation with PKC, which increases channel activity in the presence of millimolar levels of ATP. Sarc-K_{ATP} coupling to mitochondrial function through phosphotransfer system, so that changes in mitochondrial production of ATP are rapidly translated to changes in subsarcolemmal nucleotide levels, it could be of particular relevance when the mitochondrial membrane potential is depolarized, as occurs in metabolic inhibition or ischeamia. Under this conditions, mitochondrial F_0/F_1 ATPase and phosphotransfer systems can lead to a rapid depletion of ATP at the cell surface membrane and so sarc-K_{ATP} activation.[150]
The activation of sarc-K_{ATP} channel, due to hypoxia, ischaemia or pharmacological agents, accelerates the repolarisation of the myocardiocyte membrane, shortens the APD and prevents the reversal of the Na^+/K^+ exchanger, with a consequent inhibition of Ca^{2+} entry into the cell and Ca^{2+} overload, increased cell viability, readjusting the balance between energy supply and energy demand. These events can account for an increased resistance against ischaemic injury due to the activation of sarc-K_{ATP} channels; however several features then arose that raised doubts about this mechanism. In

particular, it was shown that cardioprotection was preserved in conditions where there was no APD shortening.[151,152] A number of recents reports attribute to the mito-K_{ATP} channel activation a preminent role in anti-ischaemic cardiac protection.[153-155]

This hypothesis is supported by the observation that some K_{ATP} openers, such as bimakalim and cromakalim, are able to produce cardioprotective effects also at doses devoid of any influence on APD, i.e. without affecting sarcolemmal membrane potential. This evidence suggest indirectly the exsistance of an intracellular site, independent of sarcolemmal channels, responsible for the cardioprotective action.[156-159]

The observation that, in bovine heart mitochondria, the K_{ATP} opener diazoxide induced the opening of the mito-K_{ATP} channel at concentration lower than that necessary for the opening of the sarc-K_{ATP} channel, represented the first direct suggestion about a role of mito-K_{ATP} channels.

Consistently, in isolated rat hearts, cromakalim and diazoxide, at low concentrations not affecting the sarc-K_{ATP}, produced a cardioprotective effect that is abolished by the selective mito-K_{ATP} blocker 5-hydroxydecanoate (5-HD), demonstrating the key role of this channel type.[160]

2.2.5 Mitochondrial K_{ATP} channels

Mitochondrial K_{ATP}, located in the inner mitochondrial membrane,[161] is a distinct pharmacological receptor, in particular there are some biochemical and biophysical differences between mito- and sarc-K_{ATP}. In the heart, the conductance of these channel subtypes is very different: 10 and 70-90pS, respectively. Moreover, mito-K_{ATP} is unique in its absolute requirement for Mg^{2+} for ATP inhibition and MgADP and long-chain acyl CoA esters inhibit this channel type, while they could be open by sarc-K_{ATP} (Table 2.1).

Table 2.1 Nucleotide modulation of mito-K_{ATP}

Ligand	Action	$K_{1/2}$ value
ATP (no Mg^{2+})	no effect	-
ATP (+ Mg^{2+})	inhibit	39 μM
ADP (+ Mg^{2+})	inhibit	280 μM
Palmitoyl CoA (+ Mg^{2+})	inhibit	260 μM
Oleoyl CoA (+ Mg^{2+})	inhibit	40 μM
GTP (+ATP+ Mg^{2+})	open	7 μM
GTP (+PCoA+ Mg^{2+})	open	232 μM
GDP (+ ATP+ Mg^{2+})	open	140 μM
UDP (+ATP + Mg^{2+})	open	13 μM

When cardiac mito-K_{ATP} and sarc-K_{ATP} were studied under identical conditions, diazoxide, was found to be 1000 times more potent in opening mito-K_{ATP} than sarc-K_{ATP} and 5-hydroxydecanoate (5-HD) was found to inhibit mito-K_{ATP} but not sarc-K_{ATP} Moreover, triethylammonium ion (TEA^+) inhibits most Kir subunits of the plasma membrane, but has no effect on mito-K_{ATP}.

The subunit structure of mito-K_{ATP} appears qualitatively similar to the sarc-K_{ATP}: mito-K_{ATP} also consists of two subunits: a ~ 55 kDa mitoKir and a 63 kDa mitoSUR. On the basis of its molecular size and its biochemical similarity to plasma membrane SUR, predicts that mitoSUR will turn out to be a half-molecule ABC protein. Also this channel receptor is subject to a rich variety of regulation by biochemical and pharmacological agents, some of which interact with mitoSUR, whereas others interact with mitoKir.[162]

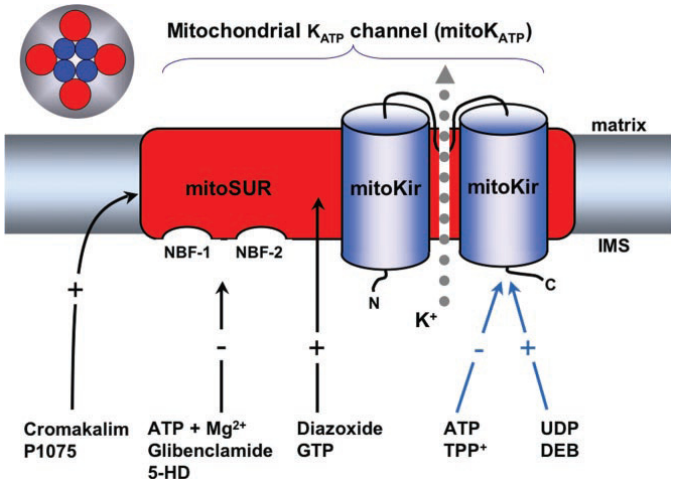

Figure 2.2 Model of the interactions of regulatory ligands with mito-K_{ATP} structure. Mito-K_{ATP} is thought to be composed of four subunits of a 55-kDa inwardly rectifying K^+ channel (mitoKIR) and at least four subunits of a 63-kDa sulfonylurea-binding regulatory subunit (mitoSUR). MitoSUR is thought to contain one or two nucleotide binding folds (NBF). ATP binds to both subunits. ATP inhibition of holo-mito-K_{ATP} exhibits an absolute requirement for Mg^{2+}, whereas ATP inhibition of mitoKIR does not, suggesting that Mg^{2+} binds to mitoSUR. GTP, which reverses ATP inhibition, interacts with mitoSUR. The classical K_{ATP} channel openers (diazoxide, cromakalim, and P1075) and blockers (glibenclamide and 5-HD) interact with mitoSUR and have no effect upon mitoKIR. The openers UDP and DEB, and the blocker TPP^+, acts upon mitoKIR.

The inner membrane potential controls the flow of K^+ ions into the mitochondrial matrix, and their leak is counterbalanced by a K^+/H^+ antiporter.
Mito-K_{ATP} plays a role in normal cell signaling processes leading to cell growth. Opening mito-K_{ATP} appears to have two distinct consequences, depending on the underlying bioenergetic state. When the membrane potential ($\Delta\psi$) is high, as in the normoxic heart, membrane permeability for ADP and ATP is quite modest and energy transfers between mitochondria and cytosol is efficiently controlled by creatine and creatine-phosphate. It has also been reported that K^+ influx via mito-K_{ATP} causes matrix alkalinization and a consequent rise in mitochondrial production of ROS.[163] The increased ROS levels, in turn, activate a variety of kinases involved in the signaling pathways

of cardioprotection against ischaemia-reperfusion injury and in pathways leading to gene transcription and cell growth. When $\Delta\psi$ is reduced, as occurs during ischaemia or inotropic stress, mito-K_{ATP} opening provides matrix volume homeostasis. In hypoxic conditions, the outer membrane conductance increases, following matrix contraction and subsequently the expansion of the intermembrane space (IMS). This change causes the dissociation of octomeric creatine kinase (CrK) from the channel and an increase in the outer membrane permeability. In this conditions, the additional K^+ influx through mito-K_{ATP}, accompanied by diffusion of water and uptake of anions (mainly phosphates and Cl^-), results in a matrix swelling, compensating for the lower driving force and maintains structure and function of the IMS volumes.[163,164]

For mitochondrial K^+ channels to play a role in preconditioning there would have to be a mechanism by which their opening might be linked to improved recovery. Several possible mechanisms have been proposed.

It is known that an increase in matrix volume can stimulate respiration and oxidative phosphorylation[165,166] and so might improve ATP production during reperfusion leading to enhanced haemodynamic recovery.[167,168] Although we were able to measure an increase in mitochondrial volume and rates of ADP-stimulated respiration in mitochondria isolated from hearts immediately following ischaemic preconditioning or diazoxide treatment, the effects were largely lost during reperfusion when they are predicted to be most important.[169] Furthermore, 5HD-treatment was shown to exhibit a similar increase in matrix volume but was not cardioprotective.[169] Garlid and colleagues[164] have proposed an alternative, but not unrelated mechanism in which it is the maintenance of the IMS and the close association between the outer an inner membrane that is the critical factor resulting from KATP channel opening .They argue that during ischaemia the loss of membrane potential would decrease electrogenic K^+ entry into mitochondria and that this would cause contraction of the matrix. It is proposed that this would disrupt the interaction between VDAC in the outer membrane, CrK in the intermembrane space and the ANT in the inner membrane leading to faster permeation of ATP through VDAC into the intermembrane space. Further translocation of this ATP into the matrix through the ANT would lead to its hydrolysis, that would be detrimental to the heart. It is suggested that opening of the mitochondrial KATP channel would prevent this matrix condensation and so decrease ATP breakdown during ischaemia leading to less damage. There is some evidence that ATP decline during ischaemia is slowed by ischaemic preconditioning or diazoxide treatment, although the mechanism originally proposed was through inhibition of the F_1/F_o ATPase by enhanced binding of the ATPase inhibitor protein.[170,171] Furthermore, other data report the opposite effect of preconditioning, with the decline in ATP and the development of contracture being faster than in control hearts.[170-173]

Garlid and colleagues further propose that during reperfusion, keeping the mitochondrial KATP channel open maintains the VDAC, CrK, ANT complex

which is vital to export ATP rapidly from the mitochondria to the cytosol where it is used to drive contraction. Although this may be true, other data showed that the slight increases in matrix volume of diazoxide-treated and ischaemic preconditioned hearts compared to control hearts was not significant at reperfusion. The more probable determinant of the efficiency of myocardial ATP production during reperfusion is the extent to which the mitochondria are damaged.[169] It has been suggested by several workers that mito-K_{ATP} opening protects the heart by uncoupling of oxidative phosphorylation due to increased K^+ cycling. Uncoupling is thought to be protective by virtue of the fact that decreased $\Delta\psi$ would reduce mitochondrial Ca^{2+} uptake and Ca^{2+} overload during ischaemia.[174-179] When the cellular energy metabolism is compromised, as in ischaemia, the intracellular Ca^{2+} levels increase (due to the entry of extracellular Ca^{2+}) and this rise of cytosolic concentration of free Ca^{2+} is partially compensated by Ca^{2+} uptake into mitochondria, mainly ensured by a highly selective ion channel known as $Ca2^+$ uniporter, and into other storage compartments. These high mitochondrial Ca^{2+} levels, togheter with other factors which are present during reperfusion (but not during ischaemia), are causally related to MPTP formation, which, during reperfusion produces Ca^{2+} release from the matrix. This opening of MPTP represents a critical cause of reperfusion-induced irreversible inury of myocardiocytes , since it leads to release of pro-apoptotic proteins.

It has been proposed that opening of K^+ channels would depolarise the mitochondria sufficiently during reperfusion to reduce ROS production and calcium accumulation and hence prevent MPTP opening.[177,180-185]

However, the amount of depolarisation predicted from K^+ channel opening is unlikely to cause much if any depolarisation[186,187] and this is confirmed by direct measurements of mitochondrial membrane potential that failed to detect any depolarisation.[188,189] Furthermore, should significant depolarisation occur at reperfusion, ATP synthesis would be compromised leading to poorer rather than better recovery of the heart. Yet NMR spectroscopy measurements have shown that the bioenergetic state of the preconditioned heart improves during reperfusion, consistent with the better haemodynamic function.[170,190,191] Nevertheless, it is not possible to rule out the possibility that opening of mito-K_{ATP} channels might cause a minor depolarisation during reperfusion, or slightly reduce the repolarisation, sufficient to reduce ROS formation without having a significant effect on ATP production. Although significant uncoupling during the reperfusion phase seems unlikely to be protective, it is known that adding low doses of uncoupler prior to ischaemia can precondition hearts.[192,193] Indeed, any interference with oxidative phosphorylation during the preischaemic phase seems able to exert a similar protective effect whether it is brought about by a brief ischaemic episode as in IPC, by addition of a respiratory chain inhibitor[194-197] or a succinate dehydrogenase inhibitor.[198] Since many of the mitochondrial K^+ channel openers have also been shown to have

direct uncoupling effects[199-202] or to inhibit components of the respiratory chain including succinate dehydrogenase,[203-205] this provides a common mode of action for these agents. How this might be translated into a protective effect at reperfusion is not clear, although a signalling pathway involving AMP-activated protein kinase provides one possibility. Another potential mechanism would be through increased levels of ROS production causing PKC activation and respiratory chain inhibitors such as antimycin are known to increase ROS production which is critical in mediating its protective effects.[194,206] It has also been reported that low doses of uncoupler can increase ROS production in isolated myocytes,[193] although how this might occur is unclear since in isolated mitochondria it is well established that even very modest uncoupling greatly decreases ROS production.[207] In the case of antimycin, PKC$_\varepsilon$ translocation has been demonstrated and its protective effects shown to be abolished in PKC$_\varepsilon$ knockout mice supporting a role for ROS and PKC$_\varepsilon$activation in its protective mechanism.[206] Nevertheless, the hypotheses about the relationship between mito-K_{ATP} activation and regulation of ROS production is quite controversial and it has been proposed, in an opposite theory, that mito-K_{ATP} channels opening might protect the heart by a further pathway: the reduction of release of reactive oxygen species linked to uncoupling of oxidative phosphorilation. It has been proposed that mito-K_{ATP} channels can acts as sensor of the mitochondrial redox balance, regulating this state under normoxic conditions and efficiently preventing the release of mitochondrial ROS and oxidative stress under pathological conditions, such as ischaemia and post-ischaemic reperfusion.

Another possible mechanism would be via an increase in matrix volume causing a stimulation of electron flow from complex 1 into complex 3.[165,208] Indeed, there are reports that low doses of valinomycin can increase ROS formation in isolated mitochondria[209] and myocytes,[164,183,210] whilst pretreatment of the perfused rabbit heart with valinomycin substantially decreased the infarct size on reperfusion.[211] However, in unpublished experiments was used Amplex Red to measured ROS production by isolated heart mitochondria incubated with a variety of respiratory substrates in State 4 and the authors were unable to detect an increase in ROS production with any mitochondrial potassium channel opener tested, or when matrix volume was increased by decreasing the osmolarity of the incubation medium. Indeed, in the latter case they detected a decrease ROS production. This is consistent with the major locus of ROS production being at a highly reduced site on complex 1 that is oxidised by an increase in matrix volume as electron flow out of complex 1 and into complex 3 is stimulated.[165,208] Another consequence of opening a mitochondrial K^+ channel would be an increase in the mitochondrial pH gradient coincident with a decrease in membrane potential.[212] Whereas the latter would be predicted to decrease ROS formation as outlined above, there is

evidence to suggest than an increase pH gradient or matrix pH can increase ROS formation from complex 1.[213] However, in view of the ability of many of the mitochondrial K_{ATP} channel openers to inhibit components of the respiratory chain,[170,186,199,203-205] its is perhaps more likely that their ability to increase ROS is through a direct interaction with a redox centre in one of the respiratory complexes.

2.2.6 Impaired glycolitic flux

Reduced blood flow and oxygen delivery to the myocardium result in metabolic and functional changes, characterized by impaired production of high-energy phosphates, intracellular acidosis, reduced developed pressure, onset of contracture, and an increased dependence on anaerobic glycolysis for energy.[18,214-216] During ischaemia, production of ATP ceases because of feedback inhibition of glycolysis by protons, lactate, and fatty acid metabolites.[18, 217,218] Numerous studies have demonstrated that maintenance of glycolytic metabolism can preserve viability in some circumstances and delay contracture, a hallmark of glycolytic failure and irreversible cell damage.[15,18,216] As a consequence of this dependence on glycolysis, conditions that impair glycolytic flux can have deleterious effects under low-flow conditions.

Some experiments have demonstrated that aldose reductase inhibition maintained higher levels of high energy phosphates and improved functional recovery on reperfusion in diabetic and control hearts subjected to low-flow ischaemia. These beneficial effects were coupled with evidence of increased glycolysis and lactate efflux, providing further evidence that increased glycolysis and lactate efflux can contribute to metabolic adaptation during low-flow ischaemia.[16] Mechanisms by which glycolysis affords beneficial effects during low-flow ischaemia are numerous. First, metabolism of glucose via glycolysis yields ATP (the process does not require oxygen), thus increasing the total amount of ATP produced under conditions of low-flow and oxygen supply. Second, although total production of ATP is clearly important in maintaining cellular function, studies suggest that the source of ATP (glycolytic vs. mitochondrial) is important for specific cellular functions and that inhibition of ATP production via glycolysis impairs the activity of Na^+/K^+-ATPase activity and the ATP-sensitive potassium channels.[219-221] The dependence of Na^+-K^+-ATPase on glycolysis has been previously established under both normoxic and hypoxic conditions.Weiss and Hiltbrand[221] have shown that inhibition of glycolysis under normoxic conditions results in significant impairment of the Na^+/K^+-ATPase. Under low-flow conditions, some studies[222,223] indicated that maintenance of Na^+/K^+-ATPase activity limited reperfusion injury. Similarly, it was demonstrated recently that ischaemia[219] in the absence of glucose resulted in more marked abnormalities in high-energy phosphates and intracellular sodium in the perfused heart.

2.3 Aldose Reductase

Aldose reductase is a monomeric, nicotinamide adenine dinucleotide phosphate (NADPH)–dependent enzyme and it is a member of the aldo–keto reductase family. This enzyme catalyzes the reduction of aldo sugars and other saturated and unsaturated aldehydes.[224-226] High levels of glucose in diabetic tissues lead to the accumulation of sorbitol via the aldose reductase pathway or polyol pathway. In this pathway, glucose is reduced to sorbitol by aldose reductase, and sorbitol is then oxidized by sorbitol dehydrogenase (SDH) to fructose. The flux through AR requires NADPH, whereas SDH requires nicotinamide adenine dinucleotide (NAD^+). Aldose reductase constitutes the first and the rate-limiting step of the polyol pathway. This pathway has been suggested to play an important role in the development of vascular and neurological complications in diabetes.[227-231] Several studies have shown improvements in neuronal, retinal, renal, and cardiac function of diabetic subjects after treatment with aldose reductase inhibitors,[227-231] suggesting that increased activity of the polyol pathway is related to many diabetic complications. Mechanisms implicated in these polyol pathway–linked functional abnormalities include myoinositol depletion and associated impaired phosphatidylinositol metabolism, osmotic imbalance due to accumulation of sorbitol, decreased availability of NADPH, and a cascade of metabolic alterations brought about by an increased ratio of free NADPH/NAD^+ resulting from increased oxidation of sorbitol to fructose by SDH in the polyol pathway.[232] Recent studies have indicated that generation of advanced glycation end products (AGEs) is facilitated by the aldose reductase pathway, thus influencing the progression of diabetic complications.[233]

Recently, was uncovered an important role for this pathway in mediating myocardial ischemic injury in diabetics. Because aldose reductase activity increases under ischemic conditions, considerable attention has been focused on studying the benefits of aldose reductase inhibition.[14-17,234]

Starting from the observation that the activity of aldose reductase increased during low-flow ischaemia, were perfomed kinetic studies in order to examine for any potential differences in catalytic activity or turnover rate between the isolated enzyme from control and ischaemic rat hearts. Data obtained[235] suggest that increased aldose reductase activity in ischaemic heart is not due to increased transcription or translation, but to activation of the enzyme by endogenous factors, such as nitric oxide (NO), which levels are increased during myocardial ischaemia, or reactive oxygen species (ROS). Pharmacological inhibition of aldose reductase significantly reduced ischaemic injury and improved functional recovery: in zopolrestat treated hearts creatine kinase (CK) release, a marker of ischaemic injury, was significantly reduced; the rise of the end diastolic pressure (EDP) during ischaemia is lower and the left ventricular developed pressure (LVDP) recovery, measured upon reperfusion, is significantly greater. In addition, aldose reductase inhibition

improves myocardial glucose metabolism. Rates of glycolysis and glucose oxidation were significantly higher in zopolrestat-perfused hearts whereas rates of palmitate oxidation remained unaffected. Under ischaemic conditions, rates of glycolysis remained significantly higher in aldose reductase-inhibited hearts. Consistent with increased glycolysis, ATP was significantly higher in aldose reductase-inhibited hearts during ischaemia. Lactate/Pyruvate (L/P) ratio, a measure of cytosolic redox state NADH/NAD^+, was increased during ischaemia and inhibition of aldose reductase with zopolrestat attenuated the increases in L/P. One mechanism by which cytosolic redox state is likely to be attenuated by aldose reductase inhibition is by attenuating the flux of substrate through sorbitol dehydrogenase (which uses NAD^+). Increases in flux via sorbitol dehydrogenase may decrease flux via glyceraldehyde-3-phosphate dehydrogenase by competing for NAD^+. L/P ratio data suggest that aldose reductase inhibition likely conserves NAD^+ and aids glycolysis by reducing NAD^+ use by sorbitol dehydrogenase.

Aldose Reductase Inhibition and Glycolysis

One possible mechanism for the observed effect of aldose reductase inhibition on glycolysis can be explained on the basis of changes in the cytosolic redox state. It has been suggested that the flux via glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a rate-limiting step for glycolysis under ischemic conditions because of increases in NADH/NAD^+ ratio.[15,18,236] Conservation of NAD^+ in any of the cytosolic pathways is likely to attenuate the increases in the cytosolic redox state. In this context, was recently demonstrated that inhibition of aldose reductase conserves NAD^+ (because of lower flux via sorbitol dehydrogenase) and lowers the NADH/NAD^+.[15] The NAD^+ conserved by aldose reductase inhibition is readily available for conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate by GAPDH[15,237] in the glycolytic pathway. Thus aldose reductase inhibition can increase glycolysis by providing NAD^+ for GAPDH.

Acidification during ischaemia.

Acidification during ischaemia results from the balance of proton production (primarily from anaerobic glycolysis and ATP hydrolysis)[238] and proton utilization either by buffering mechanisms[239] or proton efflux pathways,[240-242]Experimental data[16] suggests that acidosis during ischaemia in diabetic hearts was reduced by aldose reductase inhibition. The higher pH in aldose reductase-inhibited hearts, coupled with preservation of ATP and increased lactate release during ischaemia, is consistent with lower ATP hydrolysis and/or greater proton efflux via the lactate-H^+ cotransporter. Because reduced lactate-H^+ cotransporter activity has previously been reported in diabetics,[241] these observations, coupled with greater lactate release, suggest that aldose reductase inhibition likely improves proton efflux via this mechanism.

Recovery on reperfusion.

The rate of intracellular pH recovery on reperfusion is primarily mediated by lactate and CO_2 efflux, with smaller contributions by Na^+/H^+ exchanger and HCO_3^- influx via the Cl^-/HCO_3^- exchanger. Consistent with previous reports,[15,241] was observed[16] that pH recovery in diabetic hearts was slower than in control hearts, presumably because of impaired flux of protons via the lactate-proton cotransporter and Na^+/H^+ exchanger in diabetics.[241] As during ischaemia, normalization of pH recovery by zopolrestat further supports the postulate that aldose reductase inhibition can positively influence proton efflux mechanisms.

Aldose Reductase and Oxidative Stress

The cofactor NADPH is required for aldose reductase activity. It has been suggested that an increase in substrate flux via aldose reductase can increase oxidative stress[237,243] secondary to diminished NADPH dependent reduction of glutathione.[243] In addition to the reduction of glutathione, flux of substrate via aldose reductase can diminish nitric oxide synthase activity because of competition between these enzymes for NADPH.[244] Therefore, inhibition of aldose reductase can protect any tissue from oxidative stress. It is possible that the cardioprotection observed may, in part, be because of lowering of oxidative stress.[16].

It is clearly demonstrated that both mito-K_{ATP} openers (mito-KCOs) and aldose reductase inhibitors (ARIs) could have a fundamental role in the treatment of the myocardial ischaemia, in particular, the former may afford a "chemical preconditioning" that confers on the heart the ability to better withstand the oxygen deprivation and, consequently, to suffer less tissue damage during acute myocardial infarction; the latter as a potential therapeutic adjuncts in treating acute myocardial ischaemia and evolving infarction.

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3 KCOs and ARIs

3.1. Potassium channel openers

The potassium channel openers are a diverse group of agents originally characterized by their ability to open smooth muscle potassium channels, however their opening action is additionally exerted in a variety of tissue types, including the pancreatic β-cells, neurones, skeletal and cardiac muscle. Most of the effects of these molecules can be blocked by potassium channels inhibitors like the sulfonylurea glybencamide.

3.1.1 Chemical Classification

K_{ATP} channel openers (KCOs) exhibit chemical diversity and comprise several structural classes:

- Benzopyrans
- Cyanoguanidines
- Thioformamides
- Benzothiadiazines
- Pyridyl nitrates.

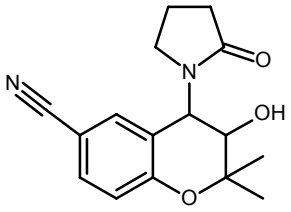
A second generation of KCOs includes:

- Cyclobutendiones
- Dihydropyridyne related structures
- Tertiary carbinols

3.1.2 Benzopyrans

Benzopyran KCOs have a broad spectrum of chemical modification and represent the most investigated chemical class of KCOs. Cromakalim[1] **1**, developed in the 1980s, is the prototype of this class.

Cromakalim contains two chiral carbons; the 3-OH group and the 4-pyrrolidinone ring are *trans*-positioned. The KCO activity resides in the 3S, 4R-enantiomer levcromakalim **2**.



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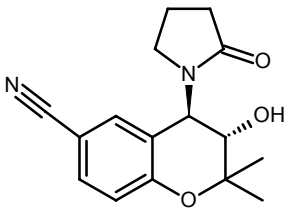

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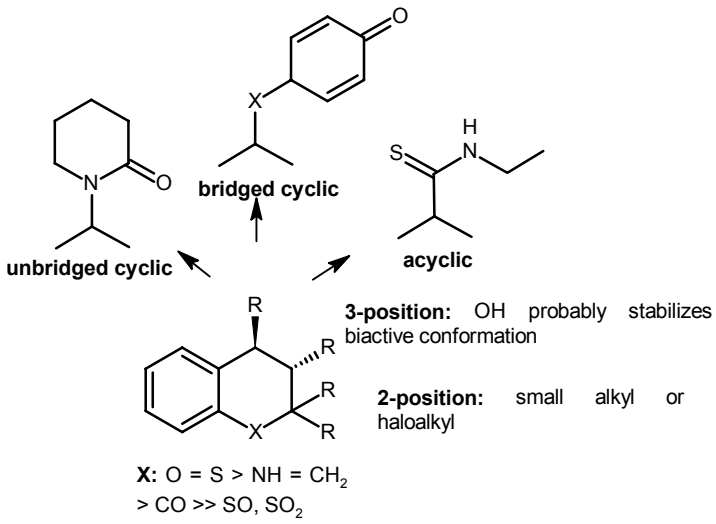


Figure 3.1 Structure activity relationship of benzopyran KCOs

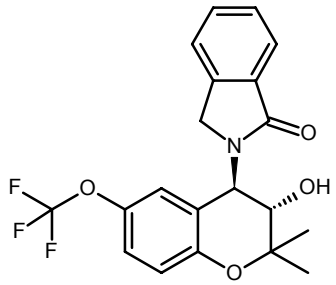
4-Position

Most extensive variations were applied to the 4-position of the benzopyran ring. The carbonyl group of the pyrrolidinyl moiety of the lead cromakalim was considered to be essential for biological activity as lactams[1] were more potent vasodilators than the original cyclic amines.[2]

Main chemical variations in 4-position comprise the insertion of:

- unbridged cyclic substituents
- bridged cyclic substituents, or
- acyclic substituents

Ring size of the unbridged cyclic substituents have a strong impact on potency with 6-membered rings exhibiting a superior potency as compared to 5-membered rings, that in turn were significantly more potent than 4-, 7- and 8-membered rings.[1,3] Additional heteroatoms,[1,4] 4-ring substituents or bicyclic replacements almost exclusively reduce potency, with the exception of celikalim **3**, which is as potent as cromakalim in lowering blood pressure. X-ray analysis of the spirocyclic compound U96501 **4** afford important informations regarding the bioactive conformation of this KCOs class: in the eutomers the carbonyl moiety points backwards with respect to the plane of the benzopyran nucleus.[5,6] The enforced orthogonality within the spirocyclic compounds supports the assumption that the bioactive conformation of freely rotable 4-substituents exhibits corresponding orthogonality.



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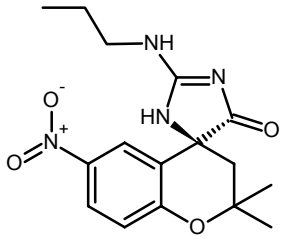
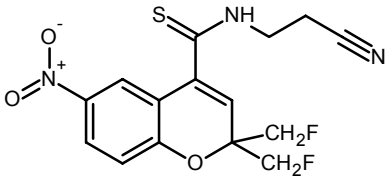


4

Linking the cyclic 4-substituent to the benzopyran ring via O- or NH-bridges often confers higher potency of these derivatives. Structure-activity relationships (SAR) for benzopyran with bridged cyclic substituents in 4-position differ from their non-bridged analogues. Positioning of the carbonyl moiety is of secondary importance. Function of the carbonyl oxygen is often mimicked by a ring nitrogen. Additional heteroatoms in the ring and ring substituent do not reduce potency. The additional methyl probably stabilizes the bioactive conformation of the bridged molecules.[7,8]

Substitution of the original lactam ring with acyclic substituents is optimal. In particular, the thioamide class includes several extremely active congeners such as KC-399 **5**,[9-12] that exhibits an *N*-β-cyanoethyl group within the thioamide function and a di-fluoromethylic substitution in 2-position.



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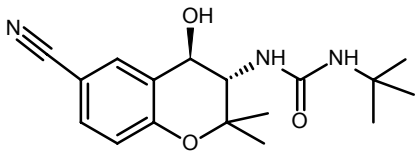
3-Position

Most benzopyrans are either unsubstituted or exhibit a hydroxy group in 3-position, replacement of this group usually diminishes antihypertensive potency. In a series of 3-variations, 3-CHO or 3-CH_2OH retained moderate potency, while 3-Br or 3-CH_3 did not possess any antihypertensive potency.[13] Some authors[14] investigated the effect of transposing the 3- and 4-substituents and compound **6** is approximately equipotent to cromakalim.

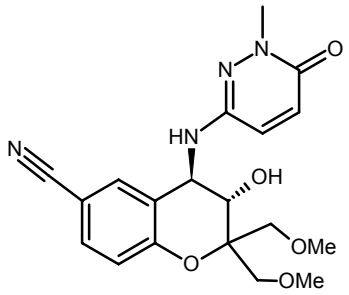


6

The importance of the 3-OH group is ambigous, whereas its presence in levcromakalim increases the potency 15-fold as compared to the 3-H analogue, chromenes such as KC-399, which lack the 3-substituent, are by far more potent than levcromakalim. Thus, 3-OH presumably does not interact with the binding site, but more probably stabilizes the bioactive conformation.[15] Only in the presence of 3-OH group the high eudismic ratio of cromakalim enantiomers is observed, whereas the eudismic ratio of the 3-H analogues is marginal.

2-Position

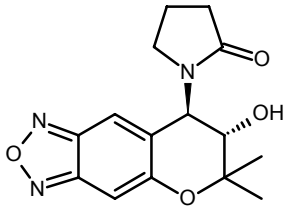
The influence of 2-substituents on the potency of benzopyran KCOs depending on the nature of the 4-substitution. For cromakalim optimal substitution is attributed to the dimethyl group, which is superior to the monomethyl and better than diethyl. The dihydro compound was virtually devoid of activity.[16] In a series of benzopyran-4-carbothioamides larger substituents are advantageous: 2-methyl-2-*n*-propyl and the spirocyclic cyclobutyl or cyclopentyl moieties yielded the most potent derivatives. Methoxymethyl substitution like in JTV-506 **7** confers selective coronary vasodilation *in vivo*.[17]



7

Aromatic Substitution

Positioning and nature of the aromatic substituents exhibit a strong influence on potency.[1,3,18] Substitution in 6-position is superior to 7-position; whereas in 5- and in 8-position abolishes activity. In early investigation, optimum substitution was attributed to small, electronegative substituents (NO_2, CF_3, CN, OCF_3, C_2F_5>MeCO>CHO>H). In NIP-121 **8** the cyanophenyl moiety is replaced by a benzoxadiazole ring.[19] Later on it was found that also larger substituents like phenyl sulfonyl are able to confer high potency.[20] Further variations[21] of 6-substitution led to 6-arylsulfonamido derivatives and 6-*N*-phenyl-*N*-methyl-sulfonamido derivative **9** is the most potent KCO among 4-pyridone–chromenes. From SAR studies and conformational analysis was postulated that 6-sulfonamido substituents extend the binding site for benzopyran KCOs, in particular beyond a site interacting with negatively polarized partial structure such us SO_2, a spatially restricted site enabling π-interactions with a phenyl moiety is assumed.



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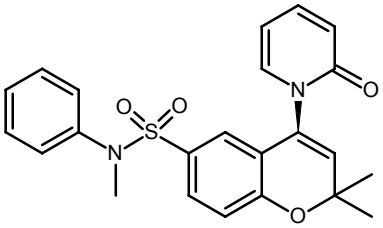
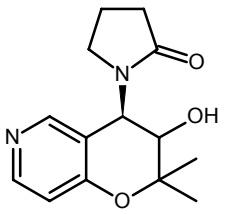


9

Transformation of the Benzopyran Nucleus

The binding site for benzopyran KCOs accomodates replacements both of the aromatic ring and of the pyran moiety. Pyridyl can replace cyanophenyl as for **10** and the pyridyl-nitrogen is preferred in position 6 instead of 7-, while 5- or 8-position are detrimental.[18]

Favourable aromatic ring modification is represented by a thiophenic ring such as in RWJ29009 **11**. Replacement of the pyran ring often yield moderately active compounds. Exchange of the pyran oxygen by sulfur represents one of the few examples of nearly retained potency.[22] Compared to cromakalim, the introduction of NH or CH_2 reduces potency by a factor of 10 and CO by a factor of 30. Oxidation to sulfoxide or sulfone is detrimental. Dialkylnaphthalenones[23] such as UR-8225 **12** are remarkable because some derivatives are selective towards tracheic K_{ATP}. Advantageous replacements of the pyran ring comprise 1,4-benzoxazine such as YM934 **13**, which is a potent vasodilator with particularly pronounced effects on coronary arteries.[24,25] The best activities was found for 1,4-benzothiazines, such as compounds **14**, **15**, which exhibit vasodilator potencies on rat aortic ring in the subnanomolar range both *in vitro* and *in vivo*.[26]



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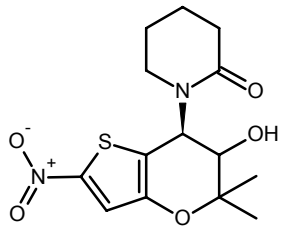

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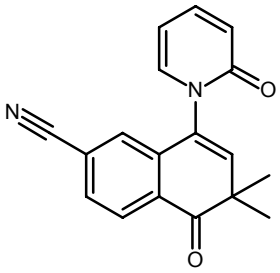

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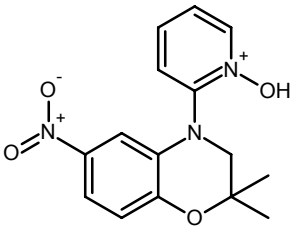

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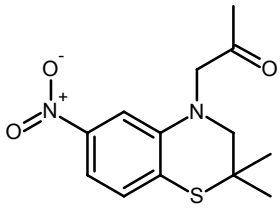

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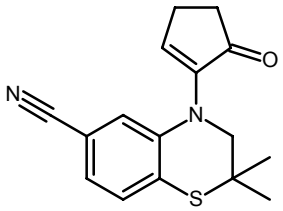
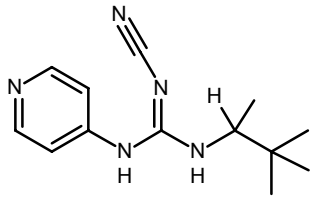


15

3.1.3 Cyanoguanidines

Prototype of the cyanoguanidines KCOs is Pinacil **16**, which was developed from a series of *N*-alchyl-*N*′- pyridyl thioureas with known hypotensive properties[27] and the KCO activity resides in the R(−) enantiomer.



16

Early SAR of pinacidil derivatives were based on *in vivo* data in spontaneously hypertensive rats. 3-Pyridyl derivatives were more potent than their 4-pyridyl

analogues except pinacidil being superior to its 3-pyridyl isomer. Exchange of the CN group in the cyanoguanidine moiety by the other polar groups resulted in compounds almost devoid of activity. In the terminal lipophilic chain region short, branched alkyl moieties proved to be optimal.
Several pharmacological studies[28-32] showed that pinacidil relaxes blood vessels by additional mechanism unrelated to K_{ATP} channel opening, thus blood pressure data are untrustworthy for SAR analyses of the KCO properties of cyanoguanidines. In consequence, some authors[33] measured the spontaneous myogenic activity and the stimulation of $^{86}Rb^{+}$ efflux from rat portal veins in order to evaluate the KCO activity of pinacidil-type cyanoguanidines, nitroethane-diamones, thioureas and ureas. A pharmacophoric model was then elaborated, which include different receptor interactions: 1) a hydrogen bond donor site, represented by the pyridyl attached NH; 2) a hydrogen bond acceptor site, represented by the pyridyl nitrogen; 3) a hydrophobic interaction site, represented by the terminal lipophilic alkyl chain.

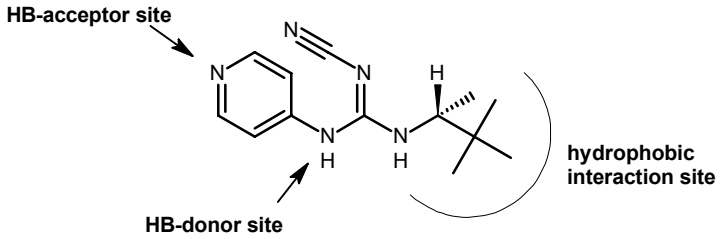
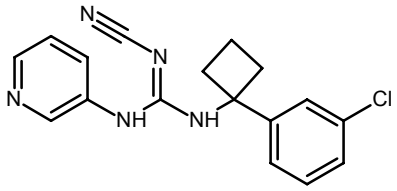


Figure3.2 Pharmacophoric model of pinacidil type KCOs

Substitution on the pyridine ring of cyanoguanidines and thioureas were investigated and substituent positioning did not significantly effect the *in vitro* potency, but maximal hypotensive response was obtained from 6-substitution. NH_2 was found the best substituent in this position and larger groups were detrimental. Corresponding with other studies, cyanoguanidines derivatives were superior to their thiourea congeners. More rigid cyclic moieties such as norbornyl derivative in the terminal lipophylic region were better than alkyl chains. SAR studies[34] on substituted phenyl cyanoguanidines showed that electron withdrawing and lipophilic substituents in 3- and 5-position confer the strongest smooth muscle relaxing activity. The pyridine ring is not essential for binding to the K_{ATP} channel and the ring attached NH and the terminal lipophilic moiety are sufficient pharmacophoric features.
The cyanoguanidines **17**, **18** are reported as K_{ATP} channel blockers. Their main chemical variation is the presence of a phenyl ring in the lipophilic side chain. Compound **17** antagonized the pinacidil-induced relation of norepinephrine contractions in isolated rabbit mesenteric arteries with an IC_{50} value of 18nM.[35] For racemic congeners, blocking activity was shown related to the (R)-enantiomers, and patch-clamp experiments and binding studies on enantiomers

of compound **18** showed a weak opener activity related to the (S)-enantiomer. It was hypothesized that it is not the ligand binding per se, but its modulation of ATP binding site and ATPase activity of the SUR subunit that determines opener or blocker properties of a ligand.[36]



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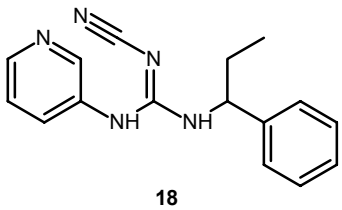

18

3.1.4 Thioformamides

Aprikalim **19** is the proptotype of this KCOs class.[37] Thioamide derivatives of tetrahydrothiophene, tetrahydrofuran, 1,3-oxathiane or 1,3-dithiane, bearing a 2-pyridyl moiety in 2 position, with known anti-ulcera and anti-secretory properties
served as precursory models. Picartamide **20**, with a 10-fold higher potency than cimetidine, and a series of derivatives were subjected to biological profiling and the 3-pyridyl isomer was found to possess marked hypotensive activity, without anti-ulcera and anti-secretory properties. Further modification on **20** revealed a superiority of the tetrahydrothiopyran over the tetrahydrothiophene ring and the antihypertensive effect could be attributed to the sulfoxide metabolite.

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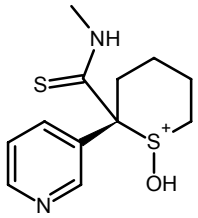

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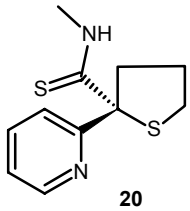

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SAR data[38,39] for this class of KCOs regard three constituting substructures:
1) the thioamide function;
2) the heterocyclic aromatic group;
3) the saturated heterocycle

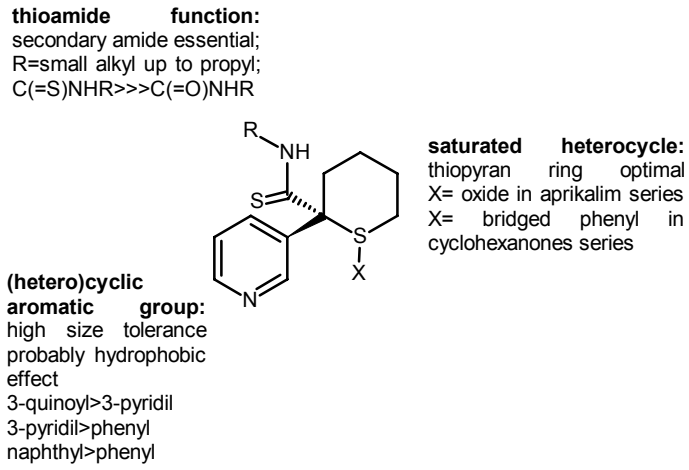


Figure3.3 SAR of thioformamide KCOs

Thioamide Function

Only very limited modification are allowed in this region of the molecule and the preference of small lipophilic group indicates the presence of a narrow hydrophobic pocket in the receptor site. Ethyl or propyl are preferred, butyl or phenyl are already exceed acceptable size, while a loss in activity is observed with the substitution of the thioamide group with an amide, indicating the precise structural requirements for the receptor interaction in this site of the molecule.

Heterocyclic Aromatic Group

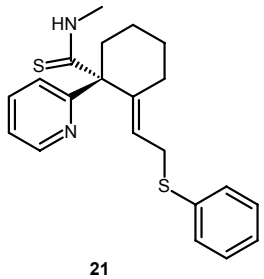
Positioning of the nitrogen in the pyridyl ring has a high impact on potency, as indicated by the loss in activity of 2-pyridyl and 4-pyridyl congeners. Also the substitution of 3-pyridyl by phenyl reduces potency, but it could be compensate by substitution with electron-withdrawing sustituents like for the 3,4-dichloro derivative.

Particularly interesting is the high potency exhibited by the 3-quinolyl for the 3-pyridyl and the superiority of the 2-naphthyl over the phenyl, indicating a higher size tolerance in the receptor site for this region of the molecule and suggesting a hydrophobic effect.

Saturated Heterocycle

Modification of the thiopyran-1-oxide group indicate that this moiety rather precisely fits the receptor demands. Sulfone, thiane, 1,3-oxathiane, 1,3-dithiane, tetrahydrothiophene and tetrahydrofuran analogues exhibit a drastic drop in activity. Only with the cyclohexane substitute the loss of activity is quite limited, so this moiety was used as lead for further chemical variation. Among the cyclohexanone analogues[40] the alkene **21**, sulfonamide **22** and the

benzylamine **23** are examples for highly potent structures. The chirally pure *trans*-benzylamine is one of the most potent known KCOs.



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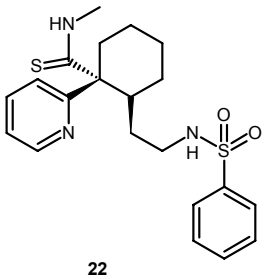

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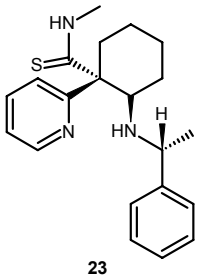

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SAR within the cyclohexanone analogues correspond with those of the aprikalim series in all features regarding enantioselectivity, absolute configuration of the eutomers, as well as modifocations of the pyridyl and thioformamide moieties. The significant gain in activity for compounds containing an additional aromatic nucleus within the 1-substituents supports the assumption of an extended pharmacophore.

3.1.5 Benzo- and Pyridothiadiazines

Prototype of the thiadiazine KCOs is diazoxide **24**, which KCO properties were determined by functional assays[41] and binding studies.[42] The binding site for diazoxide is different from that of benzopyran and cyanoguanidines[43]: it is located in other region of SUR protein. Diazoxide is the only KCO which binds with similar affinities to SUR1 and SUR2B, consequently it relaxes vascular smooth muscle and inhibits insulin secretion almost equipotently.[44]
Starting from diazoxide as a lead were developed both pancreas and smooth muscle selective derivatives.[45-48] The new structure are hybrids between diazoxide and pinacidil type KCOs and the series of pyridothiadiazine dioxides bearing a variety of 3- or 4-alkyl and 3-aminoalkyl side chains were used to elaborate a pharmacophoric model for the agonistic activity on β-cell K_{ATP} channels.

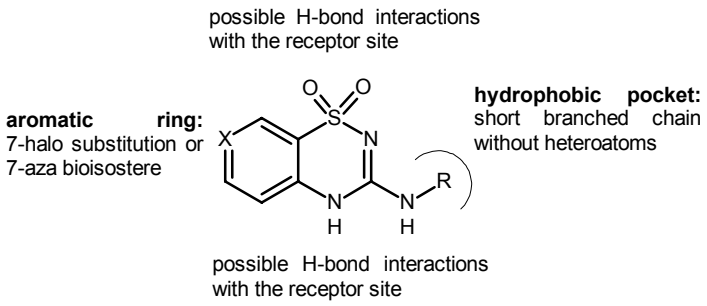
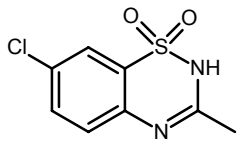


Figure3.4 Pharmacoporic model for KCO activity on pancreatic KATPchannels. Structural requirements include the presence of a short branched chain on the nitrogen in 3-position for hydrophobic receptor interactions. Hydrogen bond interactions with the receptor site are assumed for the NH goups. Also the electron-withdrawing group in 1-position could establish hydrogend bond interactions with the receptor site. Regarding the aromatic moiety, 7-halo-substituted benzene or a 7-aza-bioisostere were shown to be optimal.

In particular, the replacement of the 7-chlorobenzene moiety by the bioisosteric pyridinic nucleus significantly improves selectivity, like for the prototypical example BPDZ-44 **25**.



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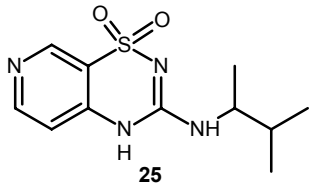

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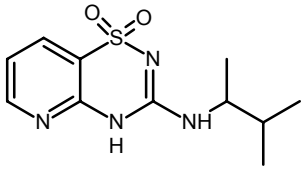
Changing the nitrogen position in the pyridine ring yields compounds with an opposite tissue selectivity: both compounds **26** and **27** were found to be more selective for relaxing the art aorta over inhibiting insulin secretion.[48] More potent and selective compounds were obtained by the replacement of the pyridine with a thiophene moiety, in particular compound **26** activates pancreatic K_{ATP} channels in the low nanomolar range and is at least 1000 times more potent than diazoxide in the insulin secretion inhibition.[49]

Hybrids between the benzothiadiazines and sulfonylureas were also synthesised and **27** exhibits vasodilator properties comparable with cromakalim.[50]



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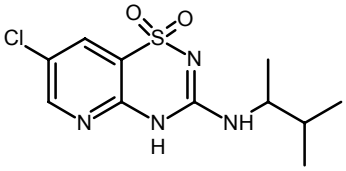

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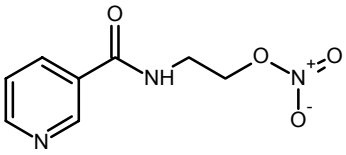
3.1.6 Pyridyl nitrates

Prototype of this class is nicorandil **28**, its vasodilator properties are due to K_{ATP} channels activation and stimulation of guanilate cyclase activity in vascular smooth muscle.[51,52]



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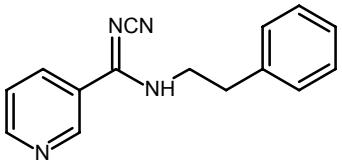

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A few of structural modifications were operated and SAR data on pyridyl nitrates are scarce. Some nicorandil analogues present the replacement of the pyridyl moiety with a piperazinyl,[53] pyrazyl[54] or thiazyl[55] ones. Compound **28** presents a cyanoamidine group instead of the amide function and an additional exchange of the nitrate group led to the pure KCO **29**.[56]

3.1.7 Benzopyranyl-Cyanoguanidines

Early studies[57] with cromakalim showed its cardioprotective effects *in vitro* and *in vivo*. Clinical efficacy, however, was limited because of equipotent systemic vasodilation.[58] A hybrid between benzopyrans and cyanoguanidines, BMS-180448 **30**, was the first derivative reported as cardioselective KCO.[59-61] BMS-180448 is not cardioselective *in vitro*; in comparison with cromakalim, however, the selectivity ratio was shifted by a factor of nearly 200 towards cardioprotective potency indicating a putative *in vivo* selectivity. Cardioprotection in the dog was observed with BMS-180448 at intravenous doses of 2.5-5 μmol/kg, whereas 21.5 μmol/kg were required to reduce blood pressure by 20%. Thus, BMS-180448 is free of hemodynamic effects at relevant cardioprotective doses. Starting from BMS-180448 Atwal *et al*.[62-66] performed detailed SAR studies to further optimize its cardioselective anti-ischemic properties.

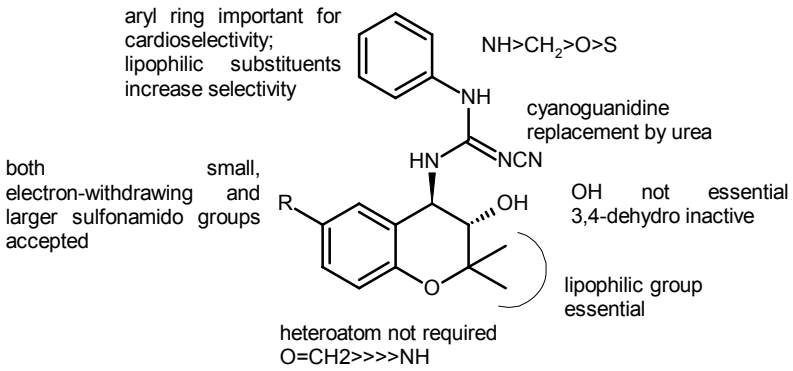
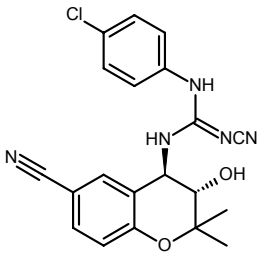


Figure3.5 SAR for the anti-ischaemic properties of the benzopyranyl-cyanoguanidine KCOs.

Regarding modifications of the benzopyran ring,27g an exchange of the oxygen in 1-position by methylene is tolerated, whereas replacement by NH is detrimental. The geminal 2-ethyl groups are essential; corresponding desmethyl analogs are devoid of activity. Presence of the 3-OH group is not mandatory, but, if present, *trans*-OH is strongly superior to *cis*-OH. Introduction of a 3,4-double bond abolishes anti-ischemic activity indicating that an sp^3 carbon is preferred at C4. In contrast, chromenes exhibit increased vasodilator properties as compared to chromanols. Thus, different SAR exist for the anti-ischemic and vasodilator properties of benzopyranyl cyanoguanidines. Urea or thiourea counterparts of the 4-cyanoguanidinyl moiety offer no advantage because they moderately improve both the anti-ischemic and the vasodilator properties. From a limited number of 6-variations, it was concluded, that small, electronegative substituents favour the anti-ischemic properties with a particularly high selectivity found for the 6-CF_3 substituted compound **31**. A later study[66] revealed larger 6-sulfonamido substituents as, for example, present in **32** to significantly improve cardioprotective potency and selectivity relative to BMS-180448.



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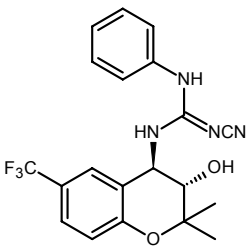

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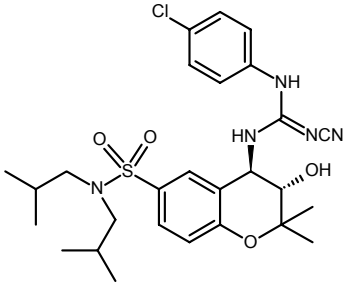
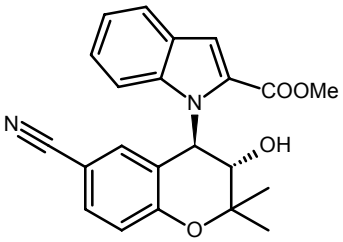


32

Modifications of the cyanoguanidine portion[63] show that the phenyl cyanoguanidine or phenyl urea moiety strongly favour cardioselectivity, since increasing or decreasing the distance between the aniline nitrogen and the pendant aromatic ring attenuates potency and selectivity. Replacements of the aniline nitrogen by O, S, or CH_2 are detrimental as well. Exchange of phenyl by 2-pyridyl or 3-pyridyl is tolerated. In addition, an advantageous phenyl substitution by small, electronegative groups such as Cl, NO_2, or CF_3 as well as small alkyl groups is found indicating favourable interactions of phenyl substituents. The impact of urea conformation on anti-ischemic activity and selectivity was evaluated via conformationally restrained analogous such as **33**. A moderate drop in anti-ischemic activity is coupled with a profound diminution of the vasodilator activity.
Attempts to further reduce the retaining vasodilator activity of BMS-180448 led the group of Atwal[65] to synthesize 4-(*N*-aryl)-substituted benzopyrans; within this series BMS-191095 **34** exhibited the highest anti-ischemic potency and selectivity. BMS-191095 is at least 30-fold more selective than BMS-180448. The anti-ischemic activity of BMS-191095 mainly resides in the 3S, 4R-enantiomer which is opposite to the enantioselectivity of BMS-180448. Reasons for this reversal of stereochemical requirements presently unknown, but may be related to different receptor binding modes. The pharmacological profile of BMS-191095 has been intensively studied *in vitro* and *in vivo*.[67-69] BMS-191095 will not be further developed for clinical use because of neuronal toxicity,[68] but it remains an interesting research tool for future drug development.



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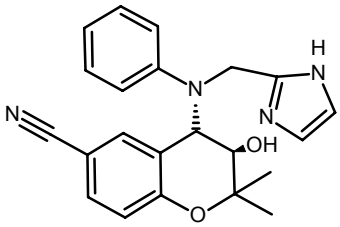

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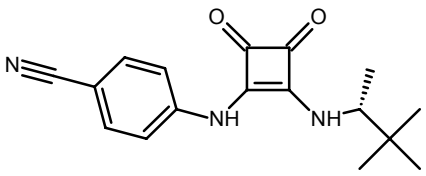
3.1.8 Diaminocyclobutenediones

Diaminocyclobutenediones, such as WAY- 133537 **35** and WAY-151616 **36** were developed at Wyeth-Ayerst.[70-72] The starting idea was to utilize the 1,2-diaminocyclobutene-3,4-dione template as a putative bioisostere for the *N*-cyanoguanidine moiety well known from the pinacidil class of KCOs.



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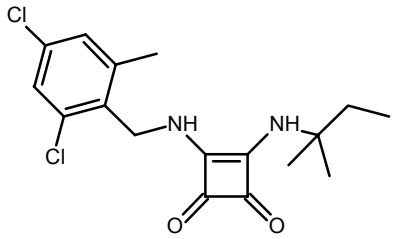

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Detailed SAR studies[70] on both the central cyclobutenedione moiety as well, as the arylamino and alkylamino side chains were reported. The central part proved to be essential. Replacing the squarate with other carbocyclic, heterocyclic or acyclic isosteres led to loss of activity. In the arylamino side chain, a phenyl group is preferred. *Ortho*-substituents enhance and *meta*-substituents reduce potency; 4-cyano is optimal. In the alkylamino side chain, rather voluminous, α-branched lipophilic groups are preferred; dilator potency resides in the R-enantiomers.

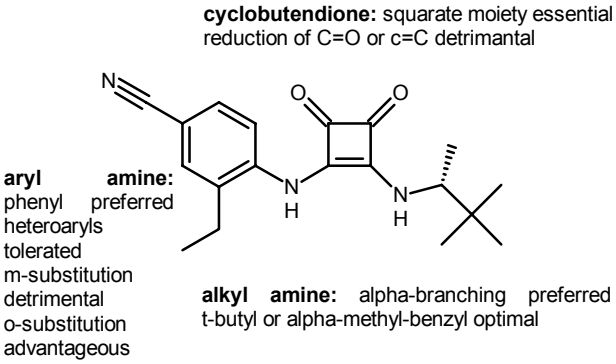


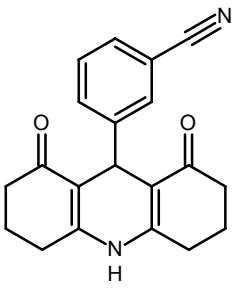
Figure3.6 SAR data of diaminocyclobutenedione KCOs.

Development candidate from these studies was WAY-133537. Efforts to improve the overall pharmacological profile of WAY-133537 were focused on metabolic stability and finally led to the design of WAY-151616.[71]
WAY-133537 and WAY-151616 relaxed rat bladder smooth muscle strips with IC 50 values of 0.09 and 0.10 μM respectively. Similar to the results with other bladder-selective KCOs (e. g., ZM-244085), WAY-133537 did not display an *in vitro* bladder selectivity versus aorta. Both compounds exhibit *in vivo* selectivity in conscious rats after oral application. WAY-133537 was 18-fold and WAY-151616 was 166-fold selective, when comparing the ED_{50} for inhibition of unstable bladder contractions and the ED_{20} for lowering mean arterial pressure.

3.1.9 DHP-Related Structures

Dihydropyridines (DHPs) are primarily known as clinically established anti-hypertensives; block of voltage-dependent L-type calcium channels, reduction of calcium entry, and relaxation of smooth muscle are the underlying mechanisms of action of their clinical use. For the enantiomers of chiral DHPs opposite molecular modes of action could be shown: the S-enantiomers of Bay-K8644 or PN 202791 are able to activate L-type calcium channels.[73] Additionally, DHPs have been shown to interact with other ionic channels. Nifedipine blocks the Ca^{2+} activated K+ transport in human erythrocytes[74] and (+)-niguldipine activates Ca^{2+} dependent, large conductance potassium (BK_{Ca}) channels in human mesenteric vascular smooth muscle.[75] Accordingly, it is not surprising that DHP-like structtues were detected which interact with KATP channels. Acridinediones were identified as putative KCOs from screening of in-house databases at Zeneca. Working hypothesis for the search was the benzopyran-analogous pharmacophoric model of an electron deficient aromatic ring in perpendicular position to a second ring containing an adjacent hydrogen bond acceptor goup. An example for DHP-like KCOs is ZM-244085 **37**.[76-78] The typical profile of the classical KCOs was also found for ZM-244085. In a subsequent study, electrophysiological measurements showed the membrane potential of the guinea-pig detrusor to be hyperpolarized 6.8 mV by 10 μM ZM-244085; in addition the compound failed to activate BK_{Ca} channels.[77] ZM-244085 possesses *in vivo* bladder selectivity.[78] Over a dose range from 0.1 to 3.0 mg/kg given orally, ZM-244085 inhibited bladder activity in conscious rats without effects on mean arterial pressure, indicating a 30-fold *in vivo* selectivity. However, ZM-244085 showed a 5.6- fold greater *in vitro* potency for the guinea pig portal vein versus guinea pig bladder strips, underlining again the poor correspondence between *in vitro* and *in vivo* data of bladder selectivity.

Replacing one of the cyclohexenone rings in ZM-244085 resulted in a further series of DHP-related KCOs including ZD-0947 **38**, A-278637 **39**, and compound **40**. ZD-0947[79]is reportedly under going clinical trials.



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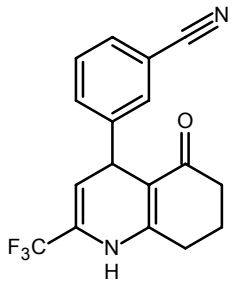

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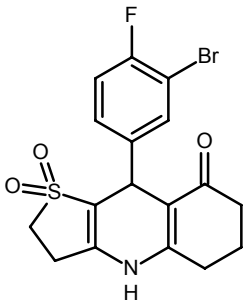

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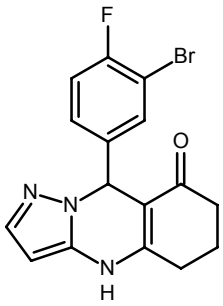

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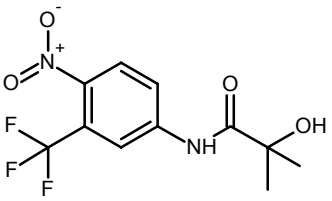
In A-278637 **39**, a thiophene 1,1 dioxide replaces one of the cyclohexenone rings present in ZM-244085. Compound **39** was subjected to a comprehensive pharmacological profiling both *in vitro* and *in vivo*.[80,81]
A novel series of tricyclic dihydropyrimidines[82] as represented by **40** deserves mentioning in the subgroup of DHP-related KCOs.

3.1.10 Tertiary Carbinols

In contrast to the above decribed KCOs, the tertiary carbinols represent entirely new chemotypes. They were derived from a series of potent anti-androgenic propanamides such as hydroxyflutamide **41**.[83] Some of these anti-androgens exhibited unwanted hypotensive effects which were later attributed to KCO properties.[84,85] First step in the structural optimization was to minimize the anti-androgen and to improve the KCO potency. A larger size tolerance for anti-androgen activity in the alkyl region and a larger size tolerance for KCO activity in the *N*-aryl region enabled this separation. In addition, KCO activity and selectivity could be shown to increase with the degree of fluorination in the alkyl region. Thus, ZM-226600 **42** is a representative of compounds with pronounced KCO, but lacking anti-androgen activity. Second step was dedicated to achieve *in vivo* bladder selectivity; the normotensive conscious rat bladder model was used as biological test system that allows the simultaneous measurement of cardiovascular and bladder effects.[86,87]



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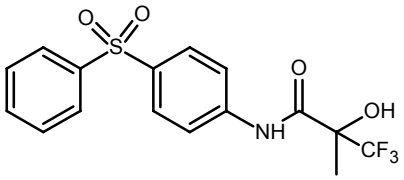

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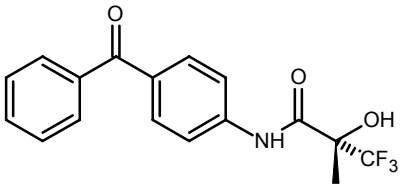
Compounds with large electron withdrawing 4- substituents as present in ZD-6169 **43** and in compound **44** exhibit rather high *in vivo* bladder selectivity, which resides in the S-(-)-enantiomers.



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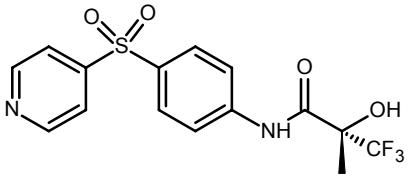
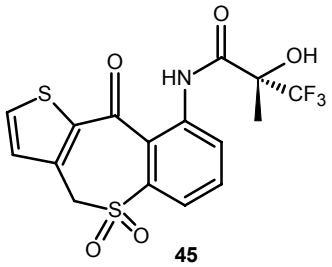


44

From these studies, ZD-6169 was selected as a development candidate for treating urge urinary incontinence. KCO properties of ZD-6169 were intensively studied *in vitro* and *in vivo*.[84-90] Chemical variations of ZD-6169 particularly include rigidization products in various parts of this lead; compounds **45-49** are the corresponding examples. The tertiary carbinols **45** and **46** differ in several ways from ZD-6I69: (i) the tertiary carbinol is ortho- instead of *para*-positioned relative to the carbonyl, and (ii) the flexible benzophenone moiety is transferred into a rigid tricyclic ring via an interconnecting seven-membered heterocycle. *In vitro*, both compounds relax 15 mM KCI- stimulated guinea-pig bladder strips (compound **45** IC_{50} = 2.1 μM and compound **46** IC_{50} = 2.5 μM) with similar potency to ZD-6169. For compounds **45** and *in vivo* bladder selectivity was reported in conscious rat cystometry at an oral dose of 3 mg/kg.[91,92]



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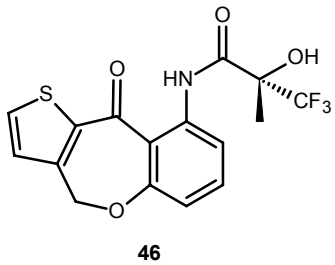
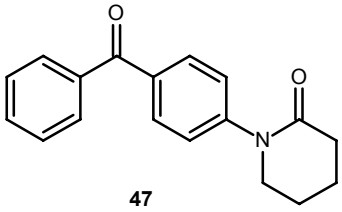


46

In structures **47** and **48**, the carbinol moiety itself is replaced by rigid rings.[93] KCO activity and selectivity of *N*-arylated derivatives of oxazolidinedione, pyrrolidin-2-one, piperidin-2-one, and morpholin-3-one were determined in vein and bladder detrusor strips of male Wistar rats. **47** (IC_{50} = 7.4 μM) and **48**

(IC_{50} = 6.7 μM) were less potent dilators of bladder detrusor strips than levcromakalim (IC_{50} = 0.34 μM), but exhibited pronounced *in vitro* bladder selectivity because of a very weak activity in rat portal veins with selectivity factors of >41 and 51, respectively.



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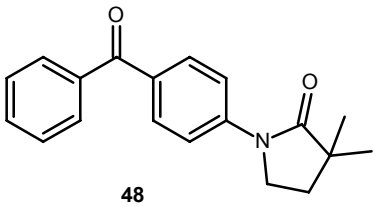
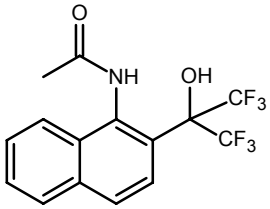


48

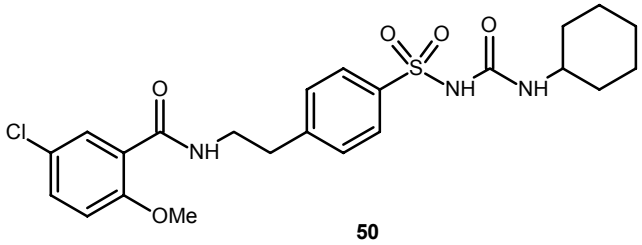
The most far-reaching modification of the lead ZD-6169 was realized in A-151892 **49**, retaining only the carbinol moiety.[94] A-151892 was shown to be a potent KCO *in vitro* in guinea-pig bladder cells and pig bladder strips. Additionally, this compound was found to selectively inhibit unstable bladder contractions *in vivo*.



49

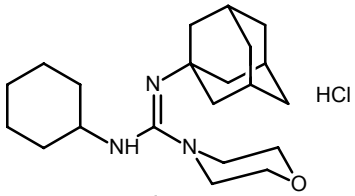
3.1.11 Potassium channel blockers

Compared to developments in K_{ATP} channel openers, medicinal chemistry research in the area of K_{ATP} blockers has seen less activity. Glibenclamide **50** has been in use since 1984 for the treatment of type II diabetes, and the pharmacology of this agent has been thoroughly described in the literature.[95]



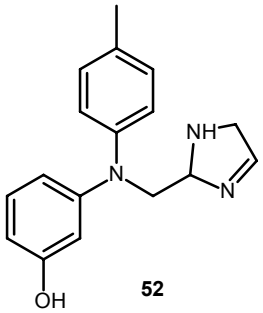
50

The vascular selective K_{ATP} blocker U-37883A **51** was shown preclinically to possess diuretic and natriuretic activity.[96] The adverse cardiovascular side effects seen with **51** in dogs and rats,[97] although not believed to be due to its K_{ATP} blocking properties, precluded its further development.



51

Other types of K_{ATP} blockers known are weak and nonselective; these include phentolamine (imidazolines), tetraethylammonium (quaternary ammonium salt), guanethidine, and bretylium. In contrast to the sulfonylurea analogues that interact at SUR1 and SUR2 proteins, phentolamine **52** and cibenzoline **53** have been suggested to directly block the channel poreforming subunit of the K_{ATP} channel.[98]



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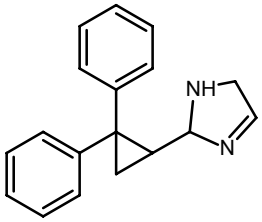
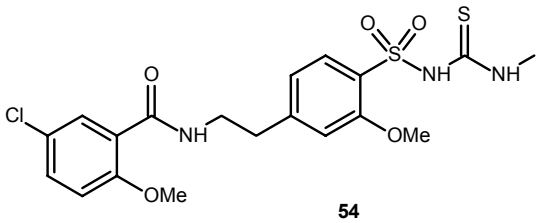


53

Considerable attention recently has been directed toward the potential of selective K_{ATP} blockers in the treatment of ventricular arrhythmias.[99] HMR-1883 **54** (clamikalant) is a novel sulfonylurea K_{ATP} blocker, currently being investigated in phase II clinical trials as the sodium salt (HMR-1098) for the treatment of ventricular arrhythmias and sudden cardiac death. HMR-1883 differs structurally from glibenclamide, in several respects: (i) exchange of the sulfonylurea for a sulfonylthiourea, (ii) the smaller size of the lipophilic substituent attached to the nitrogen of the sulfonylthiourea, (iii) the position of attachment of the sulfonylthiourea to the aromatic ring relative to the left-hand portion, and (iv) the addition of a methoxy substituent adjacent to the sulfonylthiourea. Although less potent than glibenclamide, HMR-1883 shows selectivity for cardiac K_{ATP} (guinea pig cardiomyocytes or recombinant SUR2A-Kir6.2) over the pancreatic K_{ATP} channels (rat insulinoma or recombinant SUR1-Kir6.2).

The sulfonylurea K_{ATP} channel blockers are believed to bind to a region of the SUR subunit that is adjacent to, but distinct from, that of K_{ATP} openers such cromakalim.[100,101] Indeed, the absence of obvious structural overlap between openers and blockers supports this. It is intriguing, therefore, that the potent K_{ATP} blocker PNU-99963 **55** is a member of the cyanoguanidine class. The site

of interaction of **55** is presently unknown, but this compound is an interesting new tool to probe interactions between openers and blockers at the SUR/Kir combinations.



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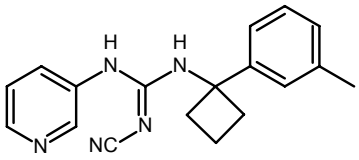
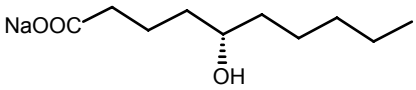


55

Sodium 5-hydroxydecanoate (5-HD) **56** is regarded as a specific inhibitor of mito-K_{ATP} channels,[102] and in several studies 5-HD has been used as a tool to test the involvement of mito- K_{ATP} in the ischaemic preconditioning.[103,104]



56

3.2 Aldose Reductase Inhibitors

Aldose reductase (alditol:NAD(P)$^+$ 1-oxidoreductase, EC 1.1.1.21) is a monomeric, nicotinamide adenine dinucleotide phosphate (NADPH)–dependent enzyme that is a member of the aldo–keto reductase family. This enzyme catalyzes the reduction of aldo sugars, including glucose, and other saturated and unsaturated aldehydes. Its crystal structure has a single domain folded into an eight-stranded parallel α/β-barrel motif, with the substrate-binding site located in a cleft at the carboxy-terminal end of the β-barrel. The coenzyme NADPH is bound in an extended conformation with the nicotinamide moiety positioned to allow a hydride transfer from the C-4 atom to the carbonyl group of the substrate while a proton is provided by the enzyme.[105,106] The pyrophosphate bridge of NADPH is tied down by loop B (residues 214-230), holding NADPH tightly in place, and loop B is fastened by the interaction of Asp 217 with lysine residues 23 and 263.[105,106] Models for the catalytic mechanism of ALR2 have recently been proposed on the basis of kinetic and crystallographic studies of wild-type and mutant forms of the enzyme. In the direction of aldehyde reduction, the enzyme follows a sequential ordered mechanism in which NADPH binds before the aldehyde substrate and NADP$^+$ is released after the alcohol product.[107,108] A kinetically detectable conformational change involving a hingelike movement of a surface loop (residues 213-217) occurs upon binding of NADPH (E·NADPH $\rightarrow$*E·NADPH)[2] and immediately prior to release of NADP$^+$.[108-110] Reorientation of this loop (*E·NADP$^+$$\rightarrow$E·NADP$^+$) to permit release of NADP$^+$ appears to represent the ratelimiting step in the catalytic mechanism.[107,108,111,112] Crystallography of human ALR2 complexed with NADP(H) revealed three residues that could potentially function as the proton donor in the catalytic mechanism: tryrosine-48, histidine-110, and cysteine-298.[109,113] Mutagenesis and structural-modeling studies, together with detailed kinetic analysis, provided compelling evidence that tyrosine-48 is most likely the proton donor.[111,112,114] Together with the nicotinamide moiety of the cofactor they compose the so-called anion-binding pocket. The composite picture of the various crystal structures suggests that the AR binding site splits into two parts with distinct flexibility properties (Figure 3.7). The part which is well superimposable across all known structures and shows virtually no variance is mainly formed by the residues of the catalytic site (Trp20, Tyr48, Val47, His110, Trp79 and Trp 111) and the flanking cofactor. Trp111 borders the anion binding pocket and exposes its π-face to the hydrophobic specificit pocket. In contrast, the second binding site portion is flexible and shows frequent, but recurring changes. Most strongly affected is the segment Val297 to Leu300 and the adjacent region composed by Trp219, Cys303 and Tyr309. To a minor extent also the residues Thr113, Phe121 and Phe122 can be involved. However, the most variable residue among all is Leu300, being the

determinant for the appearance of the occasionally formed hydrophobic binding pocket. In addition, the Cys298 to Leu300 backbone plays an important role by modulating the exposed hydrogen-bond donor and acceptor functionalities towards the ligand binding site. Additional adaptations involve Cys303, Tyr309, Thr113 and the aromatic portions of Phe115 and Phe122. Flexibility is clearly exhibited by a limited number of binding site residues: most of the mobility is transmitted by about a third of the 15 binding site amino acids. Among these, Leu300 and the backbone stretch from residue number 297 to 300 play the key role. The side-chain motions of Leu300 amplified by the backbone movements lead to significant alterations and are responsible for the major part of conformational adaptations of the binding pocket. Among the other residues, mainly Phe122 and Trp219 show the most pro-nounced mobility, both being in spatial proximity to Leu300. The molecular dynamics simulations support the dual character of the AR binding site being split into a dynamically rather rigid catalytic centre and a flexible area obviously responsible for accommodating the side chains of different substrates. In this context, it appears appropriate to think of the observed binding site adaptations not simply as a result of a ligand-induced fit, but of a preferential selection of protein conformations populated in an equilibrium.[115,116]

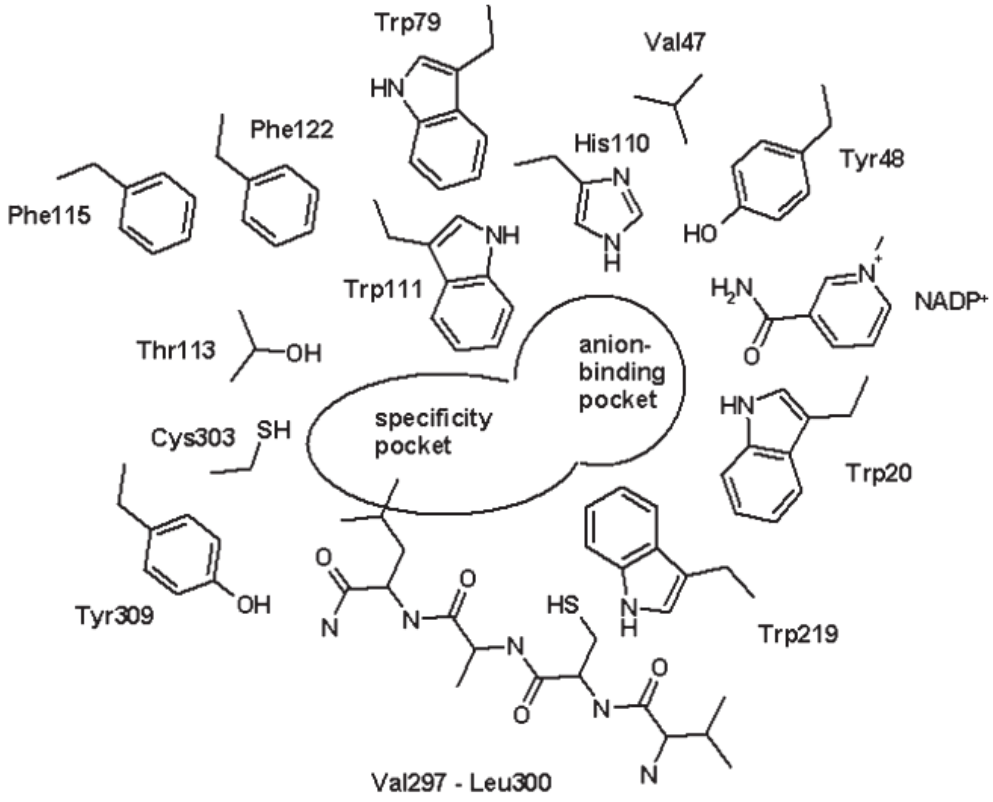


Figure 3.7 Binding site of aldose reductase

Some studies show that *in vitro* homogenous AR catalyzes the reduction of a large series of saturated and unsaturated aldehydes with 10^{-3} to 10^{-4} fold higher efficiency than glucose.[117] The enzyme is particularly efficient in reducing medium- to long chain (C-6 to C-18) aldehydes such as those generated in high abundance during lipid peroxidation.[118,119] The enzyme also catalyzes the reduction of the glutathione conjugates of unsaturated aldehydes, in many cases with efficiency higher than that of the parent free aldehyde.[120-122] Aldose reductase has a low catalytic efficiency for D-glucose, as shown by its Michaelis-Menton Costant (Km=100 mM). In normal tissue the glycaemic levels are =5 mM and the majority of glucose would be phosphorylated and flux through the polyol pathway is minimal. In diabetic patients the hexokinase pathway is saturated and the excess of glucose is shunted to the polyol pathway. The metabolic imbalance caused by the increased flux through the polyol pathway is particularly pronounced in insulin insensitive tissues. In this pathway, glucose is reduced to sorbitol by aldose reductase, and sorbitol is then oxidized by sorbitol dehydrogenase (SDH) to fructose. The flux through AR requires NADPH, whereas SDH requires nicotinamide adenine dinucleotide (NAD^+). Aldose reductase has been identified as the first enzyme involved in the polyol pathway of glucose metabolism which converts glucose into sorbitol. Glucose over-utilization through the polyol pathway has been linked by altering intracellular tonicity, generating AGEs precursors, and exposing cells to oxidative stress to tissue-based pathologies associated with diabetes complications, which make the development of potent aldose reductase inhibitors (ARIs) an obvious and attractive strategy to prevent or delay the onset and progression of the complications.

Figure3.8 Metabolism of glucose via the polyol pathway

Several generalizations have been made for the classification of AR inhibitors and some structural requirements have been identified for the enzymatic interaction:
the inhibitor must have a primary lipophilic moiety, generally an aromatic one with a polar and ionisable group, such as a carbonyl/thiocarbonyl group, located within a certain distance away from the center of the primary lipophilic moiety and a second lipophilic moiety larger that the primary one.

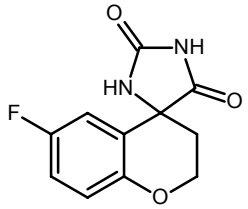
3.2.1 Chemical classification

ARIs can be categorized into six main groups:

- Cyclic imides
- Carboxylic acid derivatives
- Phenolic compounds
- Arylsulfonyl nitromethanes
- Amino acid derivatives
- Other compounds

3.2.2 Cyclic imides

Sorbinil **1**, developed in 1978, is the first cyclic imidine (spirohydantoin) compound. Several inhibition studies indicated that sorbinil may not be a successful compound for the exploration of AR enzyme inhibition within the vascular system,[123] moreover no beneficial effects on the development nephromegaly, glomerular enlargement and albumina excretion, which are common changes in renal physiology of diabetes-induced complication, have been detected. One of the main adverse effects of sorbinil is hypersensitivity reaction in the early weeks of therapy, which is most likely emerged from hydantoin moiety.



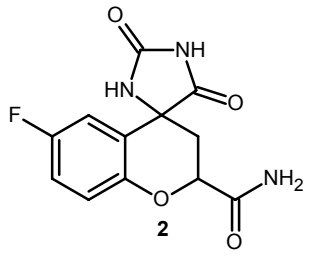
1

To solve this problem, compounds possessing thiazolidinedione moiety were introduced as hydantoin bioisoster.[124] This kind of chemical manipulation led to compounds with antioxidative effects[125] and cardioprotective properties.[126]
Sorbinil is still a main appropriate aim for bioisosteric replacements since it has chromane backbone and orthogonal spirohydantoin ring.

2-Position

Compounds derived from sorbinil present various substituent in 2-position of the chromane backbone. In a series of 2-variations, 2-fluoromethyl derivatives retained similar behaviour, 2-chloromethyl and 2-bromomethyl increases the activity 3 or 4 fold, respectively, as compared with sorbinil. The 2-CH_2OH derivative was found to be half-fold to sorbinil, while the 2-methylamino derivative showed a drastic decrease in inhibition. The 2-amido derivative fidarestat **2** increases the activity 10-fold as compared with sorbinil and is also

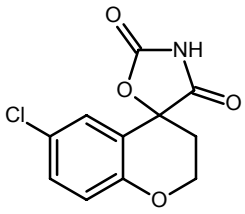
found to be more active than the halogenomethyl derivatives *in vitro*, but less active *in vivo*.[127]



2

Spirohydantoine Ring

Spirocyclic oxazolidinediones **3**, spirothiazolidinedione **4**, and spirosuccinimides **5** were synthesised in order to limit the hypersensitivity side effect linked to the hydantoine moiety of sorbinil.



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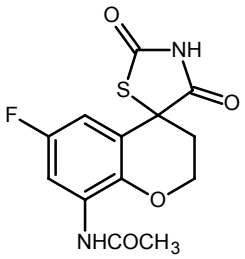

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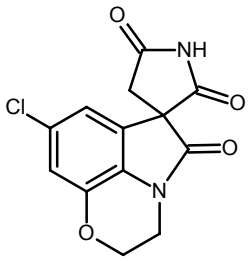
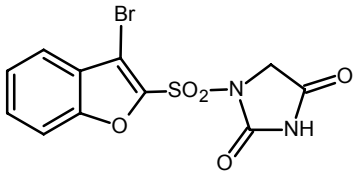


5

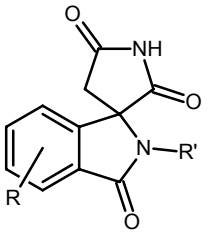
The orthogonal spirohydantoine moiety is also transferred into a more flexible one, which is bridged by sulfonyl group leading to obtain benzofuransulfonyl hydantoine **6** characterised by a potent inhibition profiles on the AR enzyme and decreased the sorbitol accumulation in isolated tissue to result in preventing experimentally induced cataracts and diabetic neuropathy.[128]



6

Chromane Ring

Incorporation of the spirosuccinimidic ring into isoindole **7** and benzisothiazole **8** resulted in marginal improvements for the inhibition.[127]



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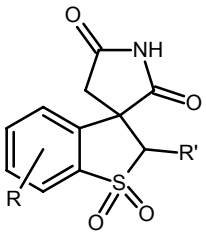
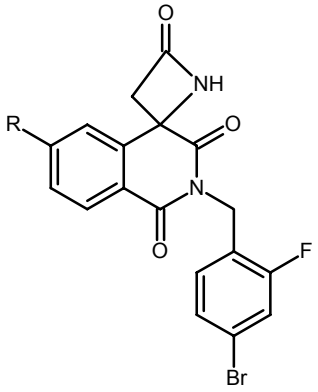


8

The chromane ring of the sorbinil was also replaced by an isoquinoline-1,3-dione moiety . In this series were also operated further modification on the spirosuccinic ring: it was replaced by an azetidine **9**, with an improvement of the orally activity, or by a piridazine one **10** with a loss of inhibitory activity.[127]



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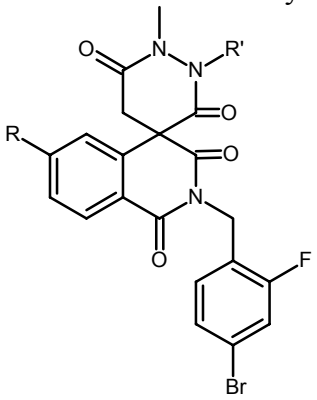
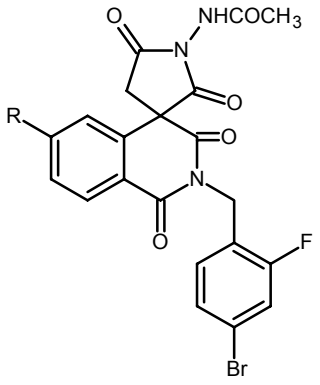


10

Finally, *N*-substituted spirosuccinimides were synthesised and the orally potency is still retained in the *N*-acetamido derivative **11**, while a loss of the *in vivo* activity was observed for the *N*-sulfonamido derivative **12**.[129]



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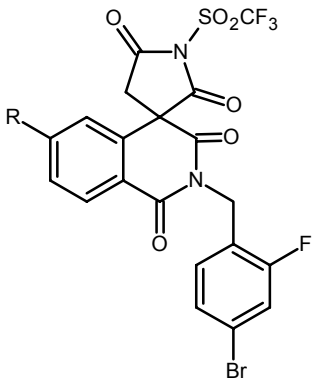
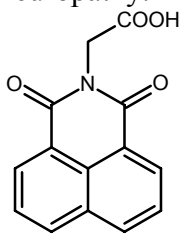


12

3.2.3 Carboxilic Acid Derivatives

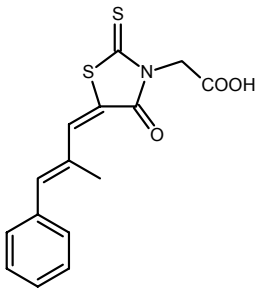
Compounds with a flexible carboxylic moiety integrated with minimal structural requirements for the AR inhibition were developed in accordance with the observation that this structural function play an important role in the interaction with the enzyme, especially in physiological condition.

Alrestatin **13** is the lead of the series and was stable when given orally and delayed the onset of diabetic complication in galactosemic rats as well as tested in diabetic patients suffering the neuropathy. [130]



13

A large number of acetic acid derivatives were also developed, including rhodanine acetic acid derivative **14**, quinolineacetic **15** and phtalazinoacetic acids **16**.



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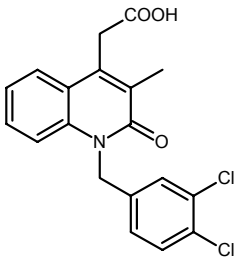
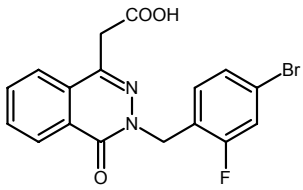


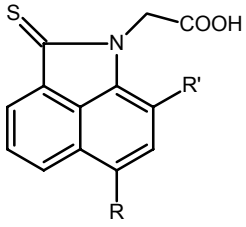
15



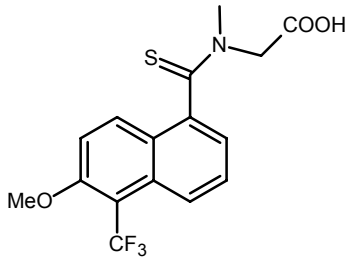
16

The clinical efficacy of epalrestat **14**, a quinolinic acetic acid derivative, was evaluated in several patients with diabetic neuropathy and showed an improvement ratings of subjective symptoms and of nerve function tests in the diabetic patients.[131] It showed also an antiproliferative antihypertophic effects on vascular smooth muscle cells induced by high glucose and suppressed intracellular NADH/NAD^+ elevation and reduced the membrane-bound protein kinase C activation.[132]

In order to develop more potent and orally active AR inhibitors, thionaphthostirylacetic acid derivatives **17** were synthesised. Although these compounds showed a good inhibitory activity both *in vitro* and *in vivo*,[133] the naphtostyril moiety produced staining of animal tissue. The cleavage of the five-membered ring of the thionaphthostyrilacetic acid moiety and several modification resulted in the tolrestat **18**.[127]

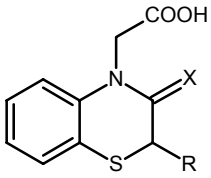


17



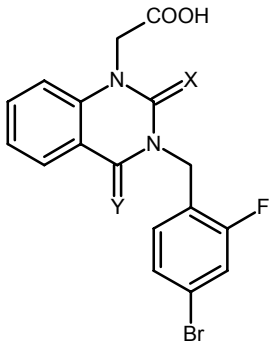
18

Oxo and/or thioxo-3-benzothiazine (**19**) derivatives were also reported as potent acetic acid ARIs.[134] Based on this findings and also from the structure of alrestatin and ponalrestat, novel ARIs having the 2,4-dioxo quinazoline ring system (**20**) were developed.[135]



oxo- or thioxo-3-benzothiazines
X=O or S

19



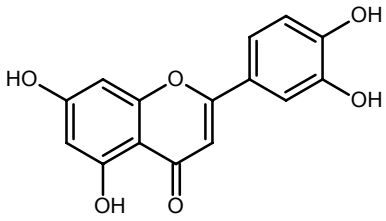
2,4-dioxo- or thioxo-quinazolines
X=O or S

20

The exchange of the acetic acid moiety with benzyl resulted in a drastic drop of activity. This may show that the flexible acid moiety might be placed in a position that could serve the interaction through the enzyme's binding site. The inclusion of halogen atoms into the benzyl ring increased the overall potency either due to their hydrogen bonding properties or the increasing lipophilicity. Besides this, methoxy group at the same ring also served the raise in activity. It is reported that these compounds showed better activity than sorbinil.[127]
Moreover, was noted that substitution of the benzyl ring greatly influenced the magnitude of the effectiveness in which electron-donating groups decreased the AR inhibition in comparison with those derivatives with electron-withdrawing group such as dihalogens. In particular, the 2-fluoro-4-bromobenzyl substitution seems to be preferred.[127]

3.2.4 Phenolic Compounds

In 1975, flavonoids were also reported as AR inhibitors in partially purified AR enzyme fron rat lens.[136] One of the most studied flavonoids is quercetin (**21**), which decresed the lens sorbitol accumulation and therefore delayed the onset of cataract formation.[137]



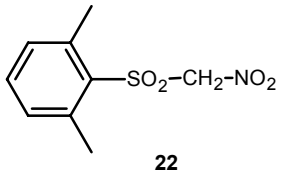
21

One of the major problems with flavonoids is their water solubility, and, therefore, they may not be affective after oral administration.[138] Since the linkage of a phenolic moiety to sugar or organic acids increase the water solubility and severely limits the passive diffusion, a number of flavonoids have been tested for their AR inhibitory activities both from natural sources and synthetic compounds.

Akin to xanthone derivatives, benzophenone ring system were also considered in the thought of possible ARIs.
The AR inhibitory activity of flavonoids and also benzophenones led to design benzopyran derivatives in search for active inhibitors for AR.[139] Some of the benzopyran derivatives showed similar inhibitory activity to that of sorbinil with possessing of selectivity for aldehyde reductase.[127]

3.2.5 Aryl Sulfonyl Nitromethanes

Compound ICI 215918 (22), was able to inhibit both AR forms, the sensitive and the insensitive ones, exhibiting similar behaviour to spirohydantoines and acetic acids.



22

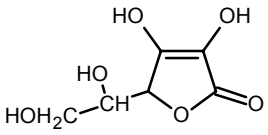
Compound **22** and one representative of spirohydantoine and acetic acids class were used in order to investigate the interaction with the binding site of the enzyme. This studies indicated a kinetic competition between these compounds, suggesting that different ARIs overlapping binding sites.[127]

3.2.6 Amino Acids

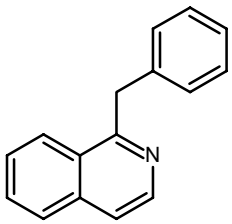
In earlier studies were synthesised *N*-glycine and alanine derivatives as AR inhibitors. Successively were synthesised *N*-benzoyl aminoacids derivatives. Inhibition analyses revealed that their kinetic mechanism of inhibition is similar, but significant differences in structure-inhibition relationship existed. For example, while the PS-alanine and the PS-2-phenylglycine showed enantioselective inhibition, no consistent pattern of enantioselectivity was observed for the isosteric *N*-benzoylalanines and phenylglycines. Also *N*-methyland *N*-phenyl substitution of PS-aminoacids did not substantially alter the inhibitory activity, while similar substitution on the *N*-benzoyl series resulted in a significant increase of the inhibitory activity. Moreover, introduction of different substituents on to *N*-benzoyl group had no appreciable potentiating effects.[127]

3.2.7 Other Compounds

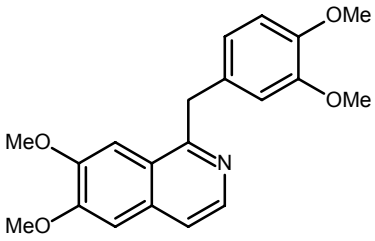
AR inhibitors from natural sources are usually involved, like the flavoids and vitamine C (**23**). Although a series of alkaloids including benzylisochinoline (**24**), papaverine(**25**) and berberine (**26**) heve been tested as ARIs, using rabbit lens, there were no satisfactory results.[140]



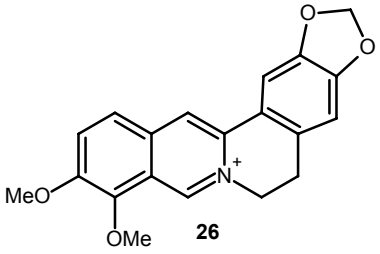
23



24



25



26

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4 INTRODUCTION TO THE EXPERIMENATAL SECTION

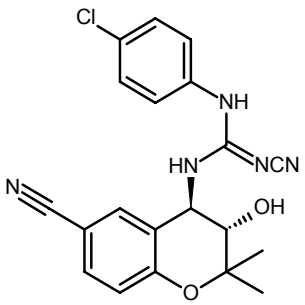
4.1 New mito-K_{ATP} channel openers

K_{ATP} channel openers (KCOs) are chemically heterogeneous molecules, which are generally lacking of receptor and tissue selectivity in particular towards cardiac mitochondrial K_{ATP} channels. Despite the strong cardioprotective activity observed in experimental studies, their clinical use for the treatment of myocardial ischaemia is limited by several side-effects, such as vasodilation and hypotension, alteration of cardiac electrophysiological mechanism and hyperglycaemia, linked to the concomitant activation of sarcolemmal K_{ATP} channels in various tissues. Moreover, pharmacological evidence suggests the implication of K_{ATP} channels in the endogenous cardioprotective mechanism known as ischaemic preconditioning (IPC). In particular, the cardiac mitochondrial K_{ATP} channels (mito-K_{ATP}) have been identified as the main effectors of IPC.[1,2]

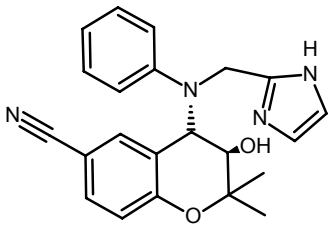
In recent years, the research was addressed to the development of new KCOs in order to find new compounds with higher selectivity toward specific targets such as the K_{ATP} of pancreatic β-cells[3] or the cardiac mito-K_{ATP} channels.[4]

The benzopyran KCOs class is the most investigated and some studies have indicated that the structure-activity relationships for the antiischaemic and vasorelaxant potencies for the benzopyran-based K_{ATP} openers are distinct.[5]

The two benzopyranyl-cyanoguanidine derivatives **BMS-180448** and **BMS-191095** have been reported to possess high cardioprotective activity linked to the mitochondrial K_{ATP} channel activation and reduced vasorelaxing properties.[6,7]



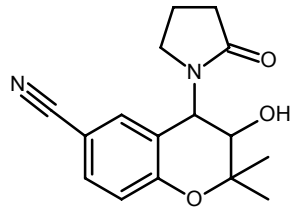
BMS-180448



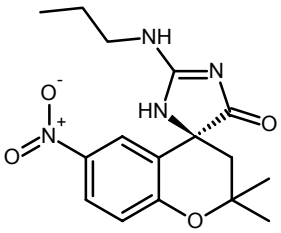
BMS-191095

Despite the large number of chemical modifications on the benzopyran scaffold, only in a few cases have been reported at the C4 position conformational rigid substituents with respect to the heterocycle-benzopyran bond, although X-ray and NMR studies on **cromakalim** have indicated that the lactam ring possess a reduced conformational freedom and prefers, for steric reasons, an orthogonally relationship with the plane of the benzopyran.

Compound **U96501**,[8] which incorporates a rigid, spirocyclic imidazolones at the benzopyran C4, shows a good activity towards K_{ATP} channels: in this molecule the spirocyclic ring fusion forces the plane of the heterocycle to be orthogonal to the benzopyran ring.

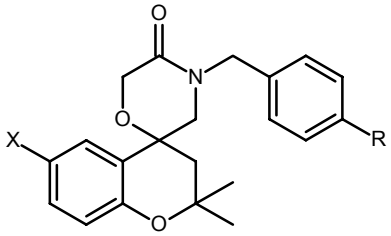


cromakalim

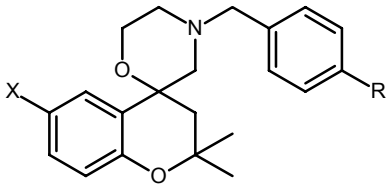


U96501

Starting from these observations, were previously synthesised new benzopyran-type derivatives in which the substituent at the C4 is represented by a spiro-morpholone (**A**) or a spiro-morpholine (**B**) ring in order to determine the effects of these structural modification on the cardioprotective properties and on the activity of these new compounds on vascular smooth muscle and blood pressure.[9]



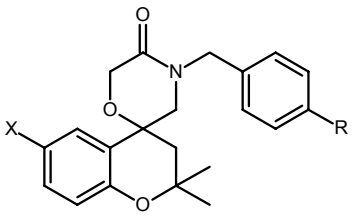
A



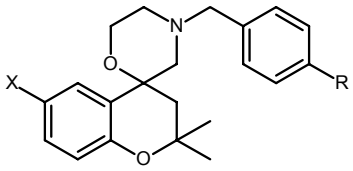
B

In particular the anti-ischaemic activity of these previously synthesised compounds was evaluated through the recording of the functional (RPP) and morphological (A_i/A_{tot}) parameters; moreover the potential role of the mito-K_{ATP} channel in cardioprotective mechanisms was investigated and the effective compounds were tested in the presence of 5-hydroxydecanoic acid (5-HD), a selective blocker of this channel type. Finally, the vasorelaxing effects *in vitro* and *in vivo* were evaluated. (Table 4.1)[9]

Table 4.1 Functional (RPP-120') and morphological (% Ischemic Area vs Total Area) parameters recorded in hearts iolated from rats pretreated with the vehicle, with the synthesized compounds or with the reference drugs, in the absence or in the presence of the selective mito-K_{ATP} blocker 5-hydroxydecanoic acid; parameters of vasorelaxing potency (pIC_{50}) and efficacy (E_{max}) of the synthesised compounds and the references K_{ATP} openers recorded in isolated rat aortic rings.



1a,b-2a,b

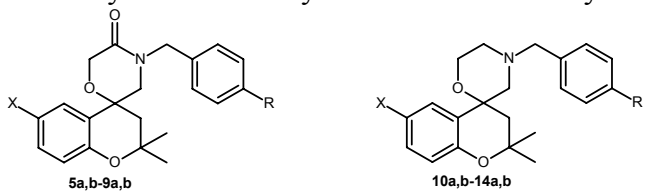


3a,b-4a,b

Compd	**X**	**R**	**RPP heart**	**120'**	**(%)**	**A_i/A_{tot} %**	**pIC_{50}**	**E_{max}%**
vehicle				23±6		35±7		
diazoxide				47±3		22±6	4.72±0.04	97±2
diazoxide + 5-HD				6±3		52±5		
(±)-cromakalim				84±16		25±1	7.01±0.09	98±1
(±)-cromakalim + 5-HD	H	NHAc		18±1		38±3		
(±)-1a				62±20		20±4	4.60±0.03	57±3
(±)-1a + 5-HD	Br	NHAc		27±11		49±5		
(±)-1b				3		58±4	4.82±0.07	70±11
(±)-1b + 5-HD	Br	NHMs		57±19		13±3	5.14±0.03	87±3
(±)-2b				39±19		47±5		
(±)-2b + 5-HD	H	NHAc		17±5		50±2	4.88±0.03	77±2
(±)-3a								
(±)-3a + 5-HD	Br	NHAc		26±4		61±2	5.62±0.03	99±1
(±)-3b								
(±)-3b + 5-HD	Br	NHMs		77±19		14±2	5.22±0.02	98±2
(±)-4b				33±16		40±1		
(±)-4b + 5-HD								

Some of these compounds showed significant anti-ischaemic cardioprotective effect devoid of vasorelaxing activity, with a pharmacological profile qualitatively similar to those exhibited by **BMS-180448** and **BMS-191095**.[9]
With the aim to investigate more deeply this new class of KCOs, were also synthesised spiromorpholone- and spiromorpholine-benzopyran derivatives with a unsubstituted benzylic moiety (**6a,b**; **11a,b**) or in which the benzylic group is substituted by an electron-withdrawing group, such as CF_3 (**8a,b**; **13a,b**), or an electron-donor substituent, such as amino (**5a,b**;**10a,b**), methyl (**7a,b**; **12a,b**) or methoxy (**9a,b**;**14a,b**)[10] (Figure 4.1).

Among the previously synthesised compounds better results in terms of both cardioprotective activity and selectivity have been showed by compound **1a**.



Compd	X	R	Compd	X	R
5a	H	NH_2	**10a**	H	NH_2
5b	Br	NH_2	**10b**	Br	NH_2
6a	H	H	**11a**	H	H
6b	Br	H	**11b**	Br	H
7a	H	Me	**12a**	H	Me
7b	Br	Me	**12b**	Br	Me
8a	H	CF_3	**13a**	H	CF_3
8b	Br	CF_3	**13b**	Br	CF_3
9a	H	OMe	**14a**	H	OMe
9b	Br	OMe	**14b**	Br	OMe

Figure 4.1 Spiromorpholones and spiromorpholines previously synthesised.

Aim of the work

In this thesis the study on this class of 4-spiro-chromane derivatives has been extended, investigating:

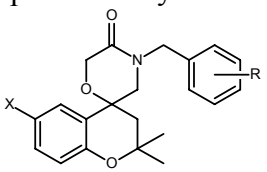
I. the influence of the position of the substituent on the benzylic group on the nitrogen atom;
II. the replacement of the benzylic moiety and the modification of the heterocyclic core;
III. the importance of the stereochemistry at the C4.

Are also reported pharmacological data obtained by a more detailed pharmacological study on the selected compound **1a**, which have shown a promising pharmacological profile.

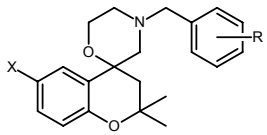
4.2 Influence of the position of the benzylic substituent

Starting from the good pharmacological profile observed for some previously synthesised compound was first investigated the importance of the position of the substituent on the benzylic moiety linked to the nitrogen atom of the spiro-morpholone and spiro-morpholine ring. In particular, was selected the methoxy-substituent for the shifting from the *para* to the *orto* and *meta* position in both type of derivatives.

Moreover, in order to extend the series, were synthesised the spiromorpholone- and spiromorpholine-derivatives in which the substituent in *para*-position of *N*-benzyl ring is represented by a bromine atom.



15a,b-17a,b



18a,b-20a,b

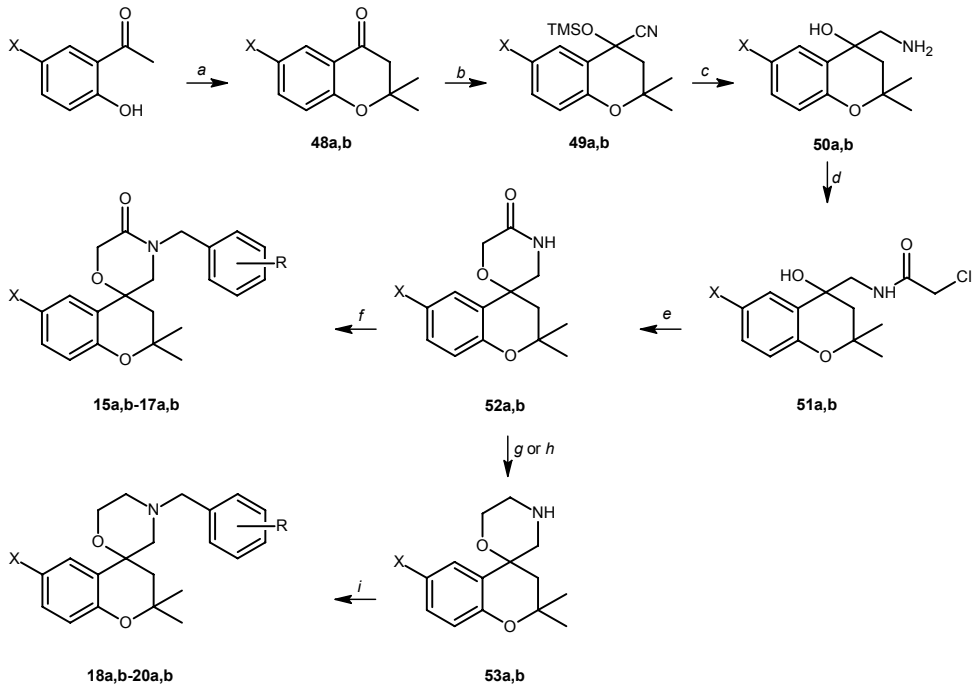
Compd	X	R	Compd	X	R
15a	H	*o*-OMe	**18a**	H	*o*-OMe
15b	Br	*o*-OMe	**18b**	Br	*o*-OMe
16a	H	*m*-OMe	**19a**	H	*m*-OMe
16b	Br	*m*-OMe	**19b**	Br	*m*-OMe
17a	H	*p*-Br	**20a**	H	*p*-Br
17b	Br	*p*-Br	**20b**	Br	*p*-Br

Synthesis

Compounds **15a,b-20a,b** were synthesised following the synthetic procedure illustrated in Scheme 4.1. Chromanones **48a,b**, obtained from the appropriate 2-hydroxyacetophenone,[11] were subjected to nucloephilic addition with trimethylsilylcyanide (TMSC) in the presence of ZnI_2 as the Lewis acid to give the corresponding trimethylsilyl cyanohydrins **49a,b**. Aminoalcohols **50a,b** are directly obtained by reduction in the presence of $LiAlH_4$ in accordance with the procedure of Amundsen and Nelson.[12] The subsequent reaction with chloroacetyl chloride in a heterogeneous phase yielded the corresponding chloroacetamides **51a,b**. Base-catalyzed (*t*-BuOK) cyclization gave the spiromorpholones **52a,b**. The subsequent reaction with the appropriate benzyl halide and NaH in DMF yielded the corresponding spiromorpholone derivatives **15a,b-17a,b**.[13] Spiromorpholine derivatives **53a**,**b** were obtained from **52a,b** by reduction with $LiAlH_4$ for **53a** or with a borane-methyl sulfide complex for **53b**.[14] This different synthetic pathway was used for **53b** in order to prevent the halogen/metal exchange that occurs in the reaction conditions.

Compounds **18a,b-20a,b** were synthesised starting from the spiromorpholines **51a,b** with appropriate benzyl halide, in the presence of K_2CO_3.

Scheme 4. 1[a]



[(a)]Reagent and conditions: (*a*) acetone, pyrrolidine; (*b)* TMSCN, ZnI_2, CH_2Cl_2; (*c*) $LiAlH_4$, THF; (*d*) chloroacetyl chloride, NaOH, H_2O/CH_2Cl_2; (*e*) t-BuOK, toluene; (*f)* benzylhalide, NaH, DMF; (*g*) $LiAlH_4$, THF or (*h*) $BH_3{\cdot}SMe_2$, THF, mw; (*i*) K_2CO_3, CH_3CN, benzylhalide.

Pharmacological results

The synthesised compound **15a,b-20a,b** were tested as racemic mixtures at a dose of 40 mg Kg^{-1} ip on Langendorff perfused rat hearts subjected to ischemia/reperfusion cycles (120 min).Two well-known K_{ATP} channel openers, diazoxide and cromakalim, were also tested as reference drugs at doses of 40 or 1 mg kg^{-1}, respectively. Diazoxide is a benzothiadiazine derivative exhibiting a preferential activity towards mitochondrial channels (at the dose commonly used in this kind of experimental protocols), while cromakalim is a very potent K_{ATP}-channel activator of both sarcolemmal and mitochondrial channels, thus possessing anti-ischaemic effects associated with strong vasorelaxing and hypotensive properties.[9,10] For each compound the ischaemic injury was evaluated by the recording of the functional and morphological parameters.

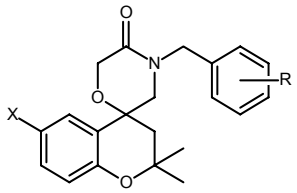
Functional Parameter

The ischaemic damage was determined through the recording of a functional post-ischaemic parameter (RPP%). In particular, the functional parameter of rate pressure product (RPP) recorded at the 120th min of reperfusion (RPP-120') has been expressed as a percentage of RPP value recorded at the last minute of the preischaemic period. This parameter was taken as indicator of the functional recovery of inotropism in the final stage of reperfusion.

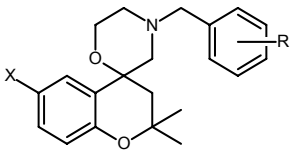
Morphological Parameter

At the end of reperfusion, the treatment of the heart with triphenyltetrazolium chloride (TTC) made it possible to carry out a morphological comparison of the necrotic and healthy areas of the left ventricular tissue, coloured white (or pale pink) and red, respectively, and then to calculate the ischemia-injured area (A_i/A_{tot}) as a percentage of the total area.

Table 4.2 Functional (RPP-120') and morphological (% Ischemic Area vs Total Area) parameters recorded in hearts isolated from rats pretreated with the vehicle, with the synthesised compounds, or with the reference drugs.



15a,b-17a,b



18a,b-20a,b

Compd	X	R	RPP 120' (%)	A_i/A_{tot} (%)	Compd	X	R	RPP 120' (%)	A_i/A_{tot} (%)
vehicle			31±4	37±4	vehicle			31±4	37±4
cromakalim			94±17	25±1	cromakalim			94±17	25±1
diazoxide			47±9	28±8	diazoxide			47±9	28±8
1a[9]	H	*p*-NHAc	62±20	20±4	**1a**[9]	H	*p*-NHAc	62±20	20±4
9a[10]	H	*p*-OMe	27±6	29±4	**14a**[10]	H	*p*-OMe	61±16	26±2
9b[10]	Br	*p*-OMe	76±19	16±4	**14b**[10]	Br	*p*-OMe	60±7	10±2
15a	H	*o*-OMe	36±19	25±5	**18a**	H	*o*-OMe	65±28	34±6
15b	Br	*o*-OMe	42±17	28±1	**18b**	Br	*o*-OMe	59±25	30±3
16a	H	*m*-OMe	29±16	41±5	**19a**	H	*m*-OMe	38±11	17±3
16b	Br	*m*-OMe	27±18	29±3	**19b**	Br	*m*-OMe	42±22	29±5
17a	H	*p*-Br	80±12	23±5	**20a**	H	*p*-Br	43±12	35±3
17b	Br	*p*-Br	23±19	47±6	**20b**	Br	*p*-Br	53±10	16±6

Data reported in Table 4.2 indicate that among the spiromorpholone derivatives only compounds **9b** and **17a** exhibited a good pharmacological profile both in terms of functional and morphological parameters. As regards the spiromorpholine analogues, while that of **9b** (**14b**) showed a good cardioprotective activity, that of **17a** (**20a**) showed a decrease of the pharmacological activity.

Discussion

As previously reported[10], the pharmacological profile of the *para*-methoxy-substituted spiromorpholones (**9a**) did not show any significant anti-ischemic activity. Change of substituent position (**15a**, **16a**) did not affect the pharmacological profile. Conversely, insertion of a bromine atom in C4′

position of *N*-benzyl ring (**17a**) led to an improvement of the pharmacological activity with inotropic recovery (RPP) better than that exhibited by **1a** (RPP = 80±12 vs 62±20) and the injured areas (A_i/A_{tot}) almost comparable (A_i/A_{tot} = 23±5 vs 20±4), indicating that an electron-withdrawing group could be a positive requirement, while the methoxy group is detrimental for the cardioprotective activity. In the limited series of 6-bromine-substituted spiromorpholones were observed opposite results: the presence of an electron-withdrawing group is detrimental, while insertion of a methoxy group in *para*-position (**9b**) was favourable for the anti-ischaemic properties. The shifting of –OMe group in position *ortho* (**15b**) and *meta* (**16b**) of the *N*-benzyl ring showed a fall in the cardioprotective activity. Also compound **17b** showed a decrease of activity.

The *para*-methoxy-substituted morpholines **14a,b** showed a good cardioprotective activity slightly lower than that of **1a** (RPP = 61±16 vs 62±20 and 60±7 vs 62±20; Ai/Atot 26±2 vs 20±4 and 10±2 vs 20±4, respectively). In this case the shifting of the substituent did not afford a significant change in the pharmacological profile both in terms of RPP and A_i/A_{tot}. For the 6-brominated derivative **20b** the insertion of bromine-atom in C4′ position of *N*-benzyl ring resulted in an improvement of the cardioprotective activity respect its spiromorpholone analogue **17b** (RPP = 53±10 vs 23±19 and Ai/Atot 16±6 vs 47±6), while compound **20a** did not show any significant cardioprotective activity.

4.3 Replacement of the benzylic moiety and modification of the heterocyclic core

In order to extend the study of the structure-activity relationships of this new class of cardioprotective benzopyran-based mito-K_{ATP} openers, have been synthesised some derivatives in which the benzylic methylene directly linked to the nitrogen atom of the spiromorpholone-and spiromorpholine-ring was replaced by a carbonyl (**C** and **E**), thiocarbonyl (**D**), or sulfonyl group (**G** and **H**). The heterocyclic core was further modified and the carbonyl of the spiromorpholones is replaced by a thiocarbonyl to produce the thiomorpholone derivatives **F** (Figure 4.2).

Compounds of type **C** still present the amidic function, but it is switched from the spirocyclic heterocycle to the nitrogen substituent. Compounds of type **D** and **F** present a thioamidic function obtained from the replacement of the carbonyl with its bioisoster thiocarbonyl. Both for the amidic derivatives **C** and for the thiomorpholones **F** were selected the benzylic substituents that have shown better cardiprotective activity.

Compounds of type **E** present the imidic function instead of the amidic one: this kind of modification led to derivatives with increased acidity. Finally,

compounds of type **G** and **H** present the sulfonyl group as additional farmacophoric group.

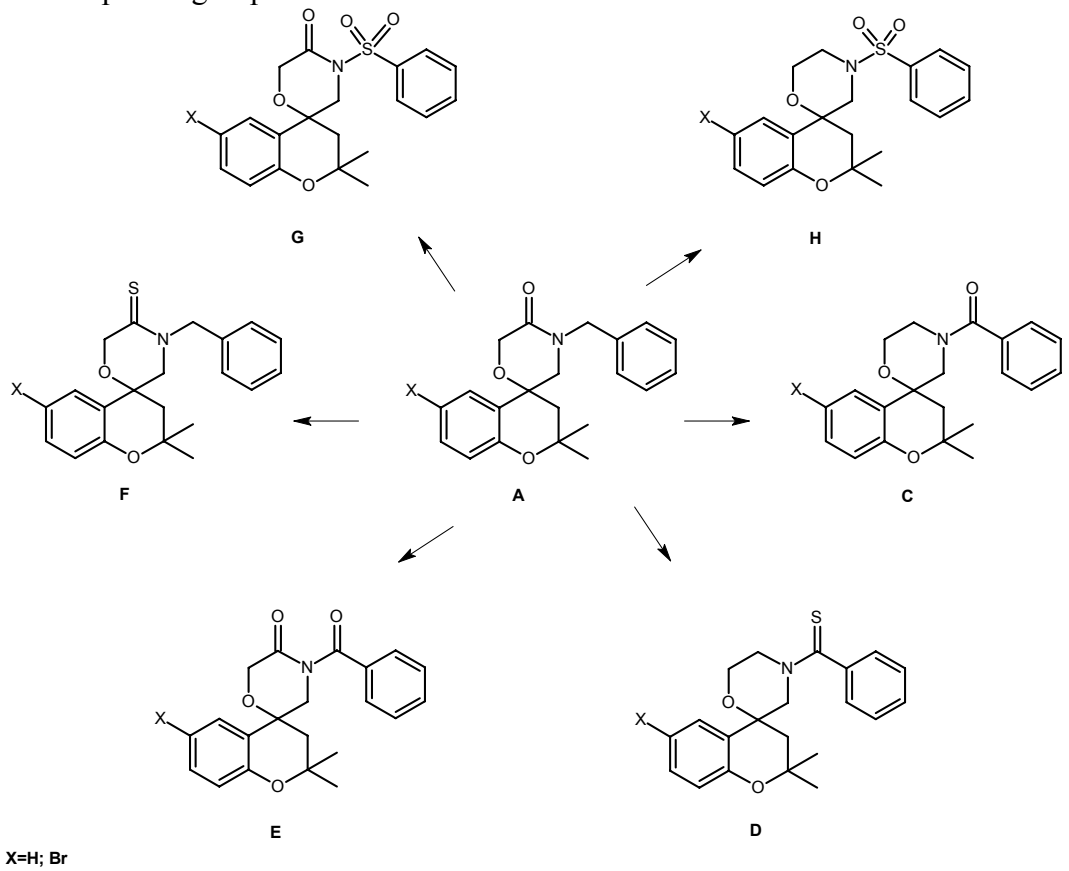


Figure 4.2 Modification of spiromorpholone and spiromorpholine scaffold.

Synthesis

Spiromorpholonone derivatives **6a,b** and **7a**, **9a** , obtained as previously described, reacted in the presence of Lawesson's Reagent[15] in chlorobenzene to give **21a,b** and **22a**, **23a**.

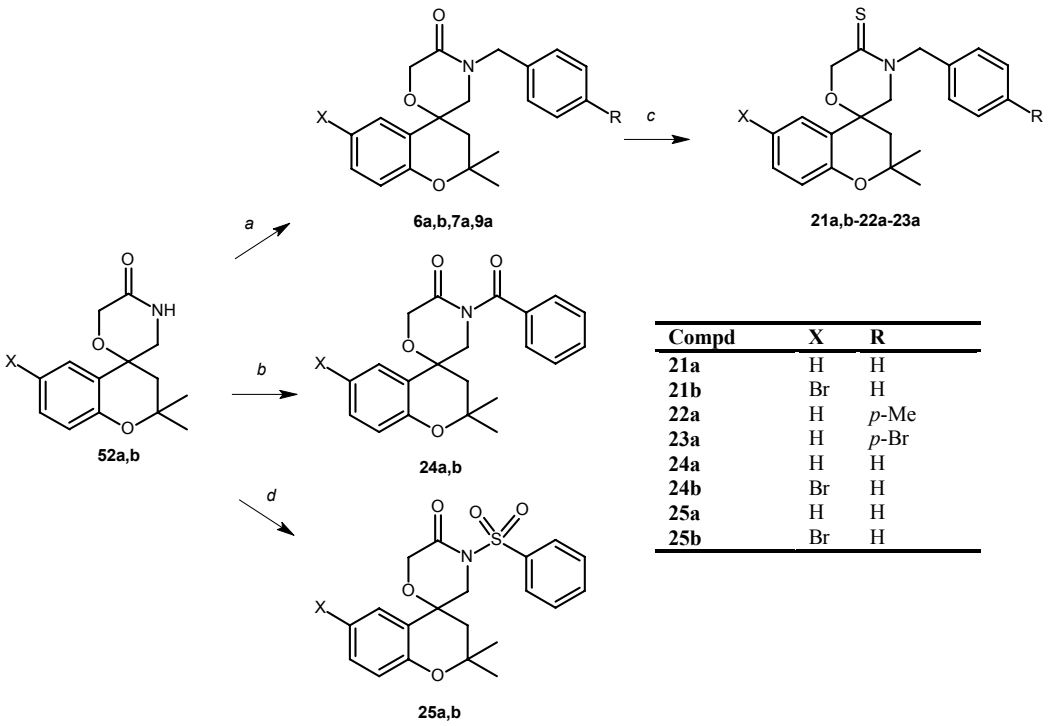
Compound **24a,b-25a,b** were obtained by reaction of **6a,b** with benzoylchloride (for **24a,b**) or benzenesulfonyl chloride (for **25a,b**) and *n*-BuLi in THF.[16]

In order to prevent the halogen/metal exchange for **25b**, particular attention during the work-up was required and the reaction mixture was quenched slowly with NH_4Cl_{sat} at -10 ˚C.

The reaction of spiromorpholines **51a,b**, obtained as previously described, with the appropriate 4-substituted benzoylchloride or benzenesulfonylchloride and triethylamine in CH_2Cl_2 yielded the corresponding *N*-benzoyl- or *N*- sulfonyl-substituted compounds **26a,b** and **27a**-**28a**. Treatment of **26a** with Lawesson's Reagent in chlorobenzene yielded **29a**.

Compound **30a** was synthesised from **51a** and *p*-aminobenzoic acid in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and a catalytic amount of 4-(dimethylamino) pyridine (DMAP). The subsequent reaction of **30a** with acetic anhydride and methanesulfonylchloride yielded the acetamido- (**31a**) and methanesulfonamido- (**32a**) compounds, respectively.

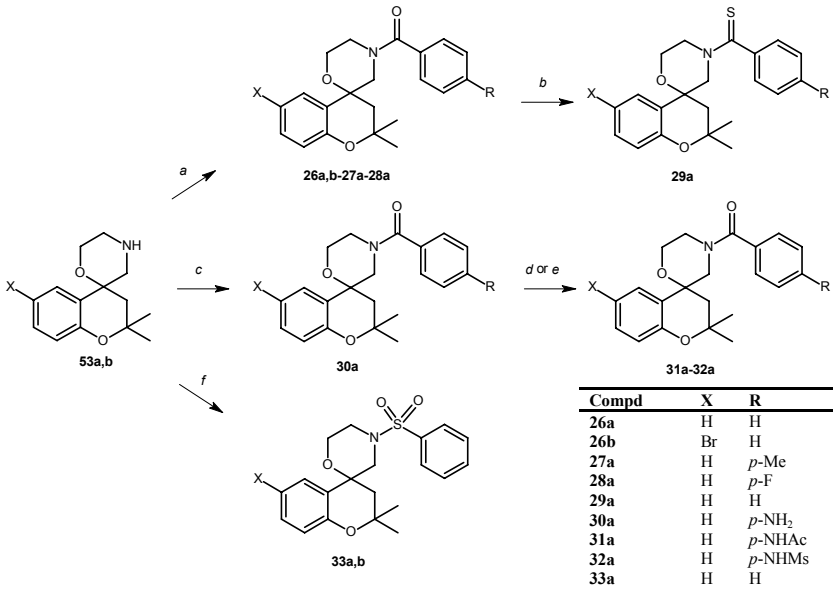
Scheme 4.2[a]



Compd	X	R
21a	H	H
21b	Br	H
22a	H	*p*-Me
23a	H	*p*-Br
24a	H	H
24b	Br	H
25a	H	H
25b	Br	H

[a] Reagent and conditions: (*a*) benzyl bromide, NaH, DMF; (*b*) benzoylchloride, *n*-BuLi, THF, N_2; (*c*) Lawesson's Reagent, chlorobenzene; (*d*) benzensulfonylchloride, *n*-BuLi, THF, N_2.

Scheme 4.3[a]



Compd	X	R
26a	H	H
26b	Br	H
27a	H	*p*-Me
28a	H	*p*-F
29a	H	H
30a	H	*p*-NH_2
31a	H	*p*-NHAc
32a	H	*p*-NHMs
33a	H	H
33b	Br	H

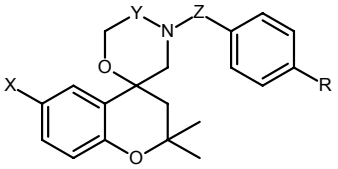
[a]Reagent and conditions: (*a*) benzoylchloride, Et_3N, CH_2Cl_2; (*b*) Lawesson's Reagent, chlorobenzene; (*c*) PABA, DCC, DMAP, CH_2Cl_2; (*e*) Ac_2O, acetone; (*d*) $MeSO_2Cl$, pyridine, dioxane; (*f*) benzenesulfonylchloride Et_3N, CH_2Cl_2.

Pharmacological Results

The synthesised compound **21a,b-33a** were tested as racemic mixtures at a dose of 40mg Kg^{-1} ip on Langendorff perfused rat hearts subjected to ischemia/reperfusion cycles (120 min); two well-known K_{ATP} channel openers, diazoxide and cromakalim, were also tested as reference drugs at doses of 40 or 1 mg kg^{-1}, respectively.

For each compound the ischaemic injury was evaluated by the recording of the functional (RPP) and morphological parameters (A_i/A_{tot}) as previously described.

Table 4.3 Functional (RPP-120') and morphological (% A_i/A_{tot}) parameters recorded in hearts isolated from rats pretreated with the vehicle, with the synthesised compounds, or with the reference drugs.



21a,b-33a,b

Compd	X	Y	Z	R	RPP 120' (%) heart	A_i/A_{tot} (%)
vehicle					20±4	37±4
cromakalim					85±9	25±1
diazoxide					55±15	28±8
1a	H	CO	CH_2	NHAc	62±20	20±4
21a	H	CS	CH_2	H	82±12	24±4
21b	Br	CS	CH_2	H	-	39±1
22a	H	CS	CH_2	Me	24±7	33±6
23a	H	CS	CH_2	Br	60±10	26±1
24a	H	CO	CO	H	38±22	42±4
24b	Br	CO	CO	H	nt	nt
25a	H	CO	SO_2	H	36±12	41±2
25b	Br	CO	SO_2	H	nt	nt
26a	H	CH_2	CO	H	69±19	43±9
26b	Br	CH_2	CO	H	44±8	31±5
27a	H	CH_2	CO	Me	-	30±1
28a	H	CH_2	CO	F	23±7	34±4
29a	H	CH_2	CS	H	nt	nt
30a	H	CH_2	CO	NH_2	nt	nt
31a	H	CH_2	CO	NHAc	61±20	16±3
32a	H	CH_2	CO	NHMs	59±6	31±1
33a	H	CH_2	SO_2	H	36	42±4
33b	Br	CH_2	SO_2	H	nt	nt

Preliminary data showed that, respect to the spiromorpholone scaffold, the replacement of the carbonyl group of the spiromorpholone with a thiocarbonyl one (**21a,b-23a**) did not improve the cardioprotective profile, with the exception of **21a**, which exhibited a good pharmacological activity, with a marked improvement of the cardioprotective activity respect its analogue **6a** (RPP 82±12 vs 35±9 and A_i/A_{tot} 24±4 vs 33±3). Both the insertion of an additional carbonyl or sulfonyl group and the inversion of the amidic function (**24a,b-33a**) did not affect the cardioprotective activity. In these limited series, the only one compound endowed of cardioprotective activity is **31a**, while compound **26a** showed a controversial behaviour. In fact, even if the RPP value of **26a** is comparable to diazoxide, the injured area (A_i/A_{tot}) is greater than that of reference drug. Conversely, for the acetamido derivative **31a** the value of the post-ischaemic functional recovery is comparable to that exhibited by diazoxide, while the necrotic area is clearly reduced. This result is completely

superimposable to this previously observed for the spiromorpholone analogue **1a**.

Discussion
Although the series of compounds is very limited, these results seems indicate that: (i) the insertion of an additional pharmacophoric group, as in compounds **25a,b**; **32a,b** is detrimental; (ii) switching the amidic function, generally, does not affect positively the pharmacological profile.

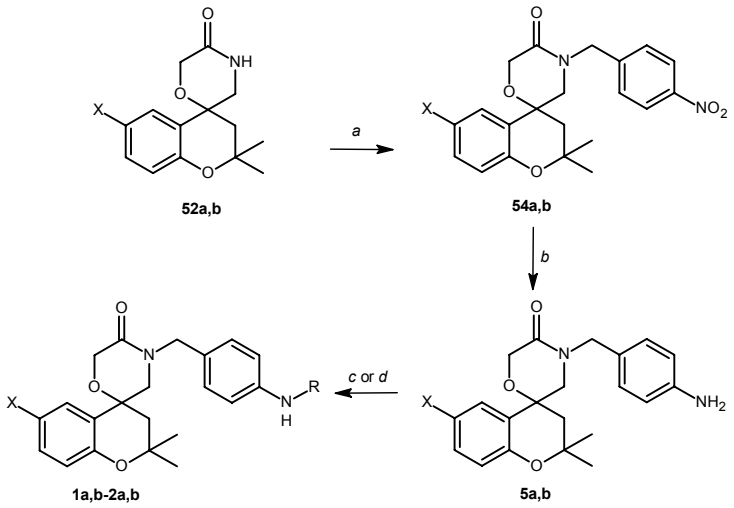
4.4 Importance of the stereochemistry

Whit the aim to understand the importance of the stereochemistry for the cardioprotective activity the 2,2-dimethyl-*N*-(4'-acetamido-benzyl)-4-spiro morpholone-chromane **1a**, which showed cardioprotective activity when administered to male Wistar rats at a dose of 40 mg kg^{1-} ip and tested on Langendorff perfused rat hearts subjected to ischemia/reperfusion cycles (120 min) was resynthesised.
Anti-ischaemic properties of **1a** could be summarized as an improvement of post-ischemic functional recovery and marked reduction of size of damaged tissue. Moreover, its cardioprotection was sensible to treatment with the selective mito-K_{ATP} inhibitor 5-HD, and this compound was devoid of significant effects on vascular smooth muscle and systolic blood pressure [9,10]
On the basis of this promising pharmacological profile exhibited in preliminary work, compound **1a** was selected for (i) the enantiomeric resolution;[17] (ii) a more detailed pharmacological study.

4.4.1 Enantiomeric Resolution

Compounds **1a,b** and **2b** were obtained following the synthetic Scheme 4.1 and 4.4, as previously reported. The nitro derivatives **54a,b** that were reduced to the corresponding amines **5a**,**b** with hydrazine hydrate in the presence of a catalytic amount of ferric chloride and activated carbon. Treatment of **5a**,**b** with acetic anhydride afforded the corresponding acetamides **1a**,**b**, while methanesulfonylation of **5a,b** gave compound **2a,b**.

Scheme 4.4[a]



[(a)]Reagent and conditions: (*a*) *p*-nitrobenzyl bromide, NaH, DMF; (*b*) $NH_2NH_2.H_2O$, $FeCl_3$, MeOH; (*c*) Ac_2O, acetone; (*d*) $MeSO_2Cl$, pyridine, dioxane.

In order to verify if the anti-ischaemic activity of this new class of 4-spiro-chromane derivatives may resides in only one or both enantiomers, the racemic mixtures of **1a** was resolved, together with that of its methanesulfonamide-analogue brominated in 6 position (**2b**), selected for comparative purposes in order to strengthen the data deriving from their racemic resolution and assignment of the absolute configurations to the single enantiomers. The direct enantioseparation of **1a** and **2b** was achieved by high performance liquid chromatography (HPLC) on chiral stationary phase (CSP). Single enantiomers, isolated at semipreparative scale, were submitted to comparative pharmacological and structural investigations. The absolute configuration assignment was accomplished by a combined strategy based on single-crystal X-ray diffraction and circular dichroism (CD) methods.
The HPLC enantioseparation of compounds **1a** and **2b** was carried out on the amylose-based Chiralpak IA CSP using normal-phase conditions. Typical chromatograms of **1a** with simultaneous UV and CD detection are showed in Figure 4.3.

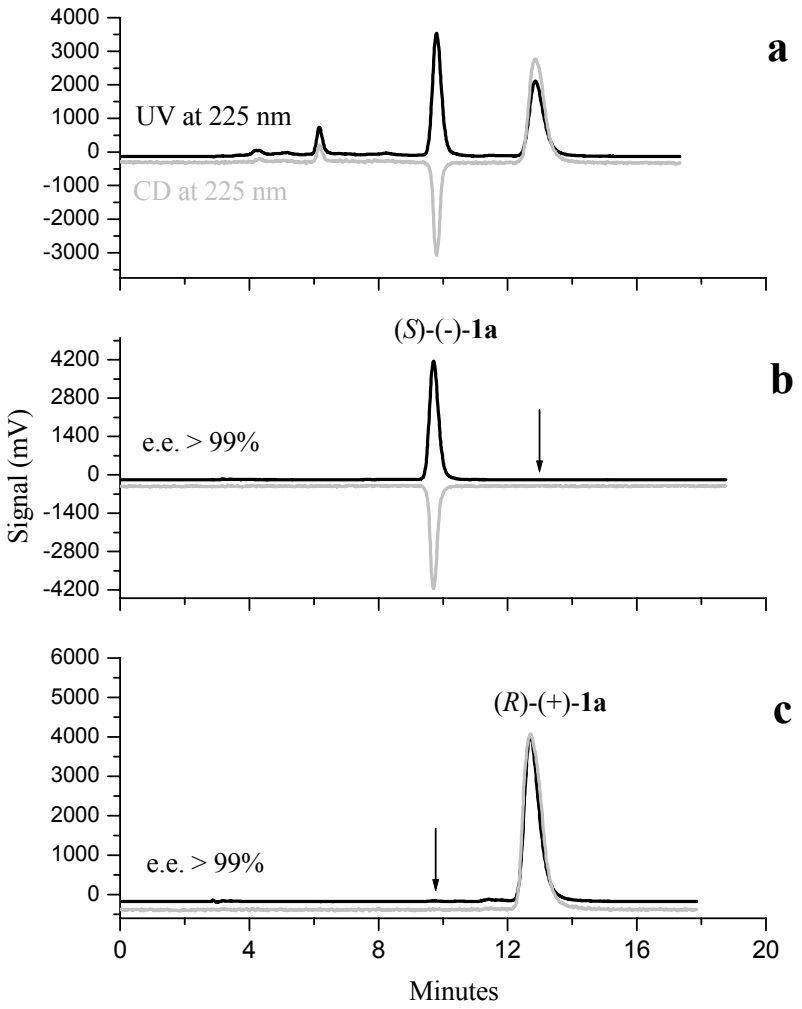
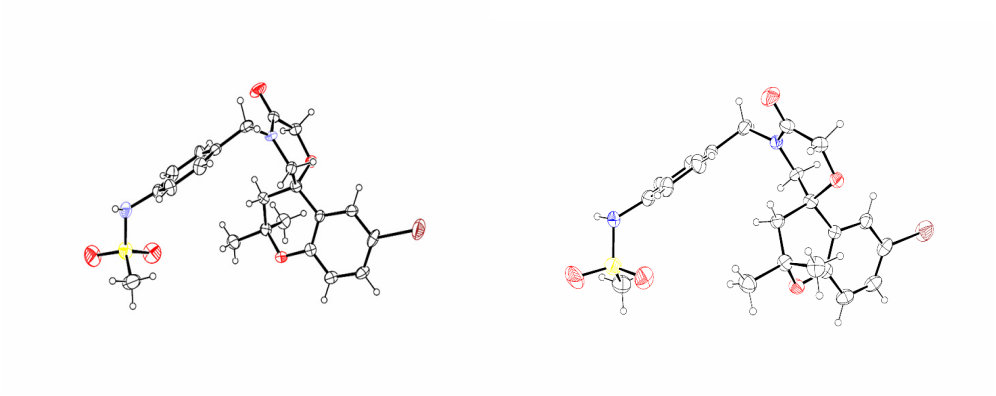


Fig. 4.3 The stereochemical correspondences between the couples of the isolated enantiomers of **1a** and **2b** were established by CD analysis.



S(-)-2b **R(+)-2b**

Figure 4.4 ORTEP drawing of (S)-(-)-**2b** and (R)-(+)-**2b**

The structures and the absolute configurations of (+)-**2b** and (-)-**2b**, and therefore of (+)-**1a** and (-)-**1a**, was readily secured by X-ray crystallography (Figure 4.4).

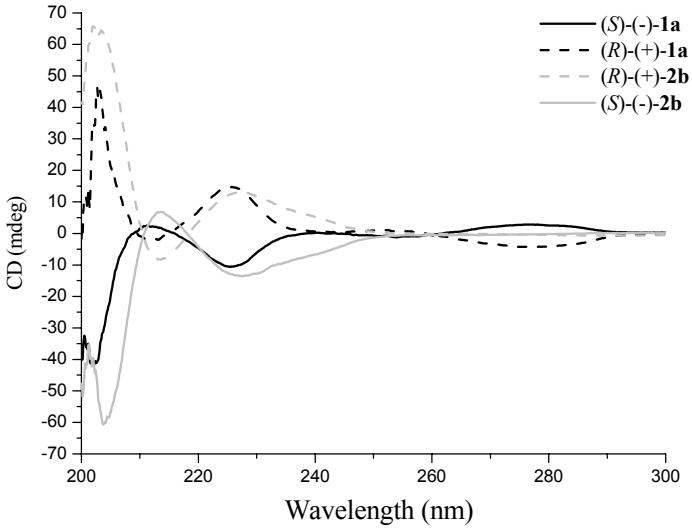


Figure 4.5 CD spectra of enantiomers of **1a** and **2b** in ethanol.

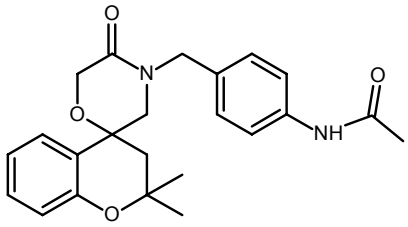
As illustrated in Figure 4.4, dextrorotatory and levorotatory enantiomers showed to have (*R*) and (*S*) configuration, respectively.

Pharmacological results

The potential cardioprotective effects of the two enantiomers of **1a** were evaluated at a dose of 40mg Kg^{-1} ip on Langendorff perfused rat hearts subjected to ischemia/reperfusion cycles (120 min); two well-known K_{ATP} channel openers, diazoxide and cromakalim, were also tested as reference drugs at doses of 40 or 1 mg kg^{-1}, respectively.

For each compound the ischaemic injury was evaluated by the recording of the functional (RPP) and morphological parameters (A_i/A_{tot}) as previously described.

Table 4.4 Functional (RPP-120') and morphological (% Ischemic Area vs Total Area) parameters recorded in hearts isolated from rats pretreated with the vehicle, with the racemate **(±)-1a**, the two enantiomers **(*R*)-(+)-1a** and **(*S*)-(-)-1a**, or with the reference drugs.



1a

Compd	RPP 120' (%)	A_i/A_{tot} (%)
vehicle	23±4	38±6
cromakalim	86±12	23±3
diazoxide	45±7	25±4
(±)-1a	59±13	22±5
(*R*)-(+)-1a	27±5	35±2
(*S*)-(-)-1a	64±20	16±6

As shown in Table 4.4, the ischemia/reperfusion cycle induced a dramatic reduction of the post-ischaemic functional parameter (RPP-120' = 21±4%) and a wide extension of the myocardial injured areas (A_i/A_{tot} = 38±6%), in hearts isolated from vehicle-treated rats. As clearly observed in previous works, the reference drugs diazoxide and cromakalim gave myocardium an increased resistance against ischemia/reperfusion, resulting in an improved recovery of post-ischaemic cardiac functionality (RPP-120' = 45±7% and 86±12%, respectively) and a marked reduction of the morphological evidence of tissue damage (A_i/A_{tot} = 25±4% and 23±2%, respectively). When administered as racemic mixture, compound **1a** confirmed its cardioprotective properties already emerged in the previous studies, with an overall amelioration of both the post-ischaemic indicators (RPP 120' = 59±13% and A_i/A_{tot} = 22±5%).

As concerns the two enantiomers of **1a**, the *(S)-(-)*-**1a** enantiomer showed significant cardioprotective effect, with a clear improvement of both the functional and the morphological markers (RPP-120'= 64±20%; A_i/A_{tot} =

16±6%). Such an anti-ischaemic activity was almost comparable to that exhibited by the racemic mixture. On the contrary, the *(R)-(+)*-**1a** enantiomer was completely devoid of anti-ischaemic effects, since the post-ischaemic functional and morphological parameters recorded in hearts isolated from animals pre-treated with *(R)-(+)*-**1a** (RPP-120'= 27±5%; A_i/A_{tot} = 35±2%) were almost superimposable to those observed in the vehicle-treated group.
In order to investigate the potential role of the mito-K_{ATP} channel in cardioprotective mechanisms, the effective compound was tested in the presence of 5-HD. The cardioprotective effects of the S-(-)-**1a** enantiomer was almost completely abolished by 5-HD, suggesting that its antiischaemic properties may be due to the activation of the mito-K_{ATP} channels.

Discussion
It is demonstrated that the interaction of the new class of anti-ischaemic spirocyclic-benzopyran derivatives with their intracellular target is enantioselective and that the cardioprotective properties of the selected compound **1a** resides in the levorotatory enantiomer while the dextrorotatory one is devoid of any activity. In addition, this result appears of particular interest because the peculiarity of such mito-K_{ATP} activators is the limited degree of conformational freedom which could let us to hypothesize a steric model for the interaction with the active site of the mito-K_{ATP} channel and therefore it could contribute to the rational design and development of a new innovative class of anti-ischaemic drugs, further improved in potency and selectivity for the cardiac target. This enantioselectivity in cardioprotection showed by this spirocyclic-benzopyran derivative is in accordance with the results obtained for the benzopyran cyanoguanidine **BMS-180447**,[18] another well-characterised activator of mito-K_{ATP} channels.

4.4.2 Pharmacological Study

In order to extend the pharmacological study of **1a** which have shown a good pharmacological profile as potential mito-K_{ATP} openers, was evaluated its anti-ischaemic effect on an *in vivo* experimental model of myocardial infarction on anaesthetized rats; the probable involvement of mito-K_{ATP} channels in a model of anoxic condition on cultured cardiomyoblasts (H9c2 cells) and, finally, the induced mitochondrial swelling.

Acute infarct
Hearts of vehicle-treated animals submitted to left anterior descending coronary artery (LAD) occlusion showed clear evidence of ischemia-reperfusion (I/R) injury, with large size of damaged tissue (Ai/Avs = 35±3%). As expected, preconditioning protocol afforded significant cardioprotective effects, producing about a half-reduction of the damaged areas (Ai/Avs = 17±6%) and

pre-treatment with diazoxide led to almost equivalent anti-ischemic effects (Ai/Avs =21±2%).
Pharmacological treatment with increasing doses (2, 10, 40 mg/Kg) of **1a,** produced significant and dose-dependent cardioprotective responses (Ai/Avs = 29±1%, 21±3% and 18±2% respectively).
When tested on 5-HD (5 mg/Kg) pre-treated animals, compound **1a** (40 mg/Kg) failed to exhibit any significant cardioprotective activity (Figure 4.6).

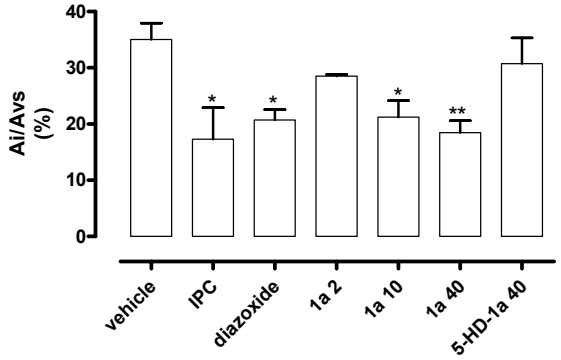


Figure 4.6 Infarct size, expressed as a percentage of the whole area of left ventricle (Ai/A_{LV}%), after different *in vivo* treatments in a model of coronary occlusion-reperfusion on rat hearts. Bars represent the Ai/A_{LV}% of the hearts of rats that 2 hours before the experimental procedures received vehicle or respectively an i.p. injection of: diazoxide 40 mg/Kg, compound **1a** 2 mg/Kg, compound **1a** 10 mg/Kg, compound **1a** 40 mg/Kg and 5-HD 5 mg/Kg plus compound **1a** 40 mg/Kg. The asterisks indicate a value significantly different from the vehicle (* = P< 0.05; ** = P< 0.01).

H9c2 cell viability

The incubation in anoxic environment (95% N_2 and 5% CO_2) caused a drastic reduction of absorbance values taked as an index of H9c2 cell viability (Viab.% = 62 ± 5). Pre-treatment of cells with diazoxide (100 μM) and **1a** (10 μM) conferred a strong resistance, producing a full preservation of cell viability (Viab.% = 108±4 and 106 ± 1, respectively). 5-HD (500μM) antagonised the protective effects of both diazoxide and **1a** (Viab.% = 58 ± 5 and 63 ± 4, respectively) (Figure 4.7).

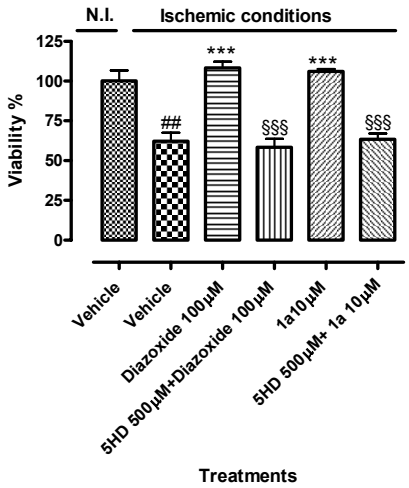


Figure 4.7. Cell viability normalised as percentage of the reference value, i.e. the non-ischemic vehicle-treated cells (N.I.). The bars represent the H9c2 viability after the indicated treatments. The symbol # indicates a value of viability significantly different from the reference value N.I. (## = P< 0.01). The symbol * means a value of viability significantly different from the vehicle-treated ischemic cells (*** = P< 0.001). The symbol § means statistical differences from the corresponding value obtained in the absence of 5-HD (§§§ = P< 0.001).

Mitochondrial swelling

The spectrophotometrical evaluation of the influence of the tested compounds on matrix volume showed that both diazoxide and compound **1a** evoked mitochondrial swelling. In particular, the decrease in absorbance at 520 nm recorded 15 min after the addition of diazoxide 100 µM or **1a** 100 µM were 0.110±0.013 and 0.123±0.014 (in absorbance arbitrary units), respectively (Figure 4.8). Compound **1a** 10 µM did not produce any decrease of absorbance (data not shown).

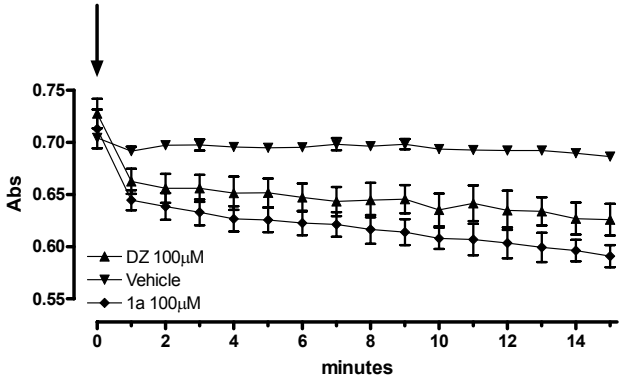


Figure 4.8 Rat heart mitochondrial swelling represented by the decrease in absorbance at 520nm recorded for 15 minutes after the addition (arrow) of vehicle, diazoxide 100μM and compound **1a** 100μM.

Discussion

Compound **1a** was tested *in vivo*, in an experimental model of acute infarct. In this model, more closely resembling the complex clinical pattern of the heart infarct, compound **1a** showed cardioprotective effects almost comparable to those produced by the exogenous "self-defence" mechanism of IPC. Moreover, the anti-ischemic effects of **1a** showed a dose-dependent fashion and were equivalent (or slightly superior) to those of the reference drug diazoxide. As already observed in the previous studieson isolated hearts,[9] the cardioprotective effects of **1a** were inhibited by 5-HD, selective blocker of mito-K_{ATP} channels. The probable involvement of mito-K_{ATP} channels clearly emerged also from the experimental protocols on cultured H9c2 cells. In this model of cardiomyoblasts, the anoxic condition caused a dramatic impact, leading to a high level of cell death. Both diazoxide and compound **1a** (added of a concentration 10-fold lower than the reference benzothiadiazine) produced powerful protective effects, leading to a complete prevention of cell death induced by the incubation in the anoxic atmosphere. Again, the mito-K_{ATP} blocker 5-HD fully antagonised the protective effects.

Given the strong indications by the above results about a mito-K_{ATP} activating mechanism of action, the studies were focused on the identification of "diagnostic" effects of compound **1a** at the mitochondrial level, i.e. effects comparable to those produced by the reference mito-K_{ATP} activator diazoxide.

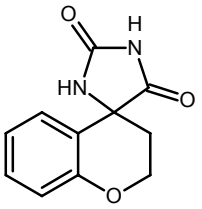
Movements of K^+ ions across the inner mitochondrial membrane, such as their influx into the matrix through electrogenic mechanisms (K^+ channels and K^+ leak) and their extrusion mainly due to K^+/H^+ exchanger, are fundamentally involved in the homeostatic control of matrix volume.[19-21] In particular the opening of K^+ channels leads to a K^+ influx exceeding the K^+ extrusion, which is compensated by increased H^+ outward pumping and accumulation of phosphates in the matrix. This causes osmotic influx of water and mitochondrial swelling.[21,22] In agreement with its pharmacodynamic feature of K^+ channel activator, diazoxide caused mitochondrial swelling.

A quantitatively and qualitatively similar effect was produced by compound **1a**, but only at a concentration 10-fold higher (100 μM) than that exhibiting protective effects on H9c2 cells.

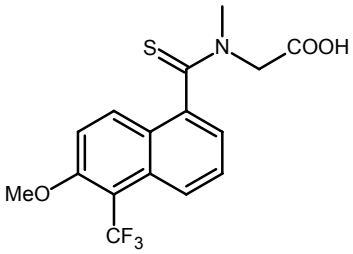
4.5 New aldose reductase inhibitors

Aldose reductase (ALR2) and aldehyde reductase (ALR1) belong to the aldo-keto reductase superfamily of enzymes whose members are responsible for a wide variety of biological functions. Aldose reductase has been identified as the first enzyme involved in the polyol pathway of glucose metabolism which converts glucose into sorbitol. Glucose over-utilization through the polyol pathway has been linked to tissue-based pathologies associated with diabetes complications, which make the development of potent aldose reductase inhibitors (ARIs) an obvious and attractive strategy to prevent or delay the onset and progression of the complications. Recently, it was demonstrated that inhibition of AR protects rat hearts from ischaemic injury and provide a foundation for evaluating aldose reductase inhibitors as potential therapeutic adjuncts in treating patients with myocardial infarction.

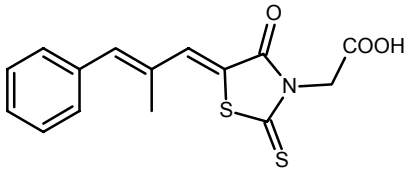
ARIs are generically distinct in two main classes: spirohydantoines, such as **sorbinil**, and acetic acid derivatives, such as **tolrestat**, **epalrestat**, **zopolrestat** and **ponalrestat**.



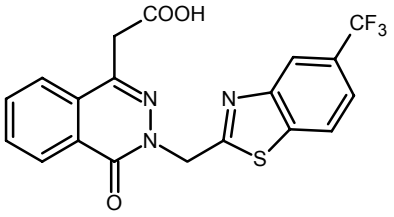
Sorbinil



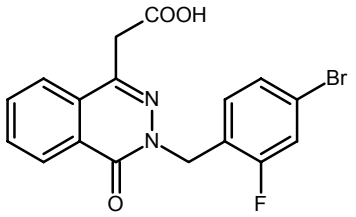
Tolrestat



Epalrestat



Zopolrestat

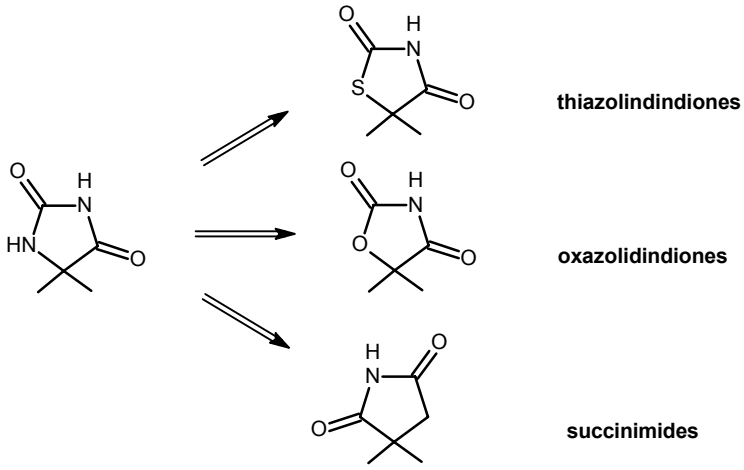


Ponalrestat

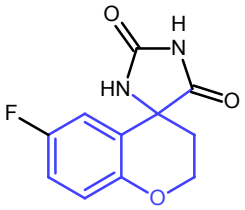
Both classes possess the structural requirements for the enzymatic interaction: an acidic group for the interaction with the anionic site and a lipophilic group for interaction with the hydrophobic pocket, fundamental for the activity and selectivity of the enzyme. ARIs of the carboxylic class like zopolrestat are quite selective for aldose reductase vs aldehyde reductase and other enzymes. Howevever these carboxylic class of agent becomes highly protein bound *in vivo* thus limiting their efficacy *in vivo*. Hydantoin ARIs like sorbinil, on the

other hand, are relatively non-selective and inhibit aldehyde and aldose reductase with comparable efficacies.[23]

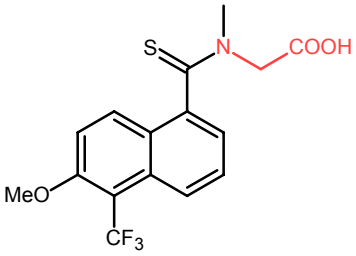
The well known ARI sorbinil presents a benzopyran nucleus in which the C4 is involved in a spirohydantoine cycle. This compound has not therapeutic utility because of side-effect, such as hypersensitivity reaction, linked to hydantoinic core. Many chemical modifications have been reported in which the imidic cycle is bioisosterically replaced by thiazolidindiones, oxazolidindiones and succinimides.



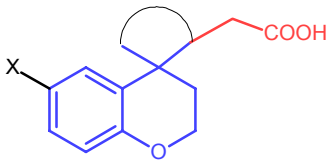
SAR and computational studies have revealed that a flexible acidic group, such as an acetic chain, linked to an amidic function is important for the interaction with the anionic site of the enzyme.



Sorbinil

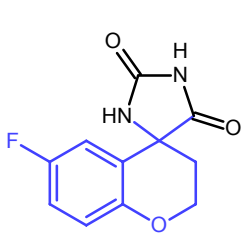


Tolrestat

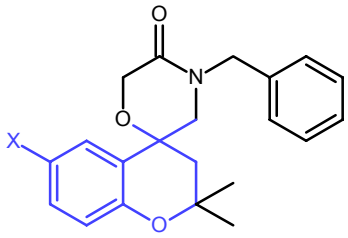


I

Starting from the structural analogies existing between ARI sorbinil and the new 4-spiro-chromane derivatives synthesised potassium as mito-K_{ATP} channel openers (**A**), a part of this thesis focused on the development of new ARIs.

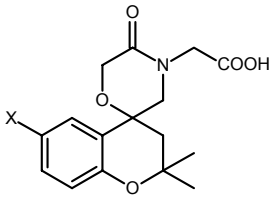


Sorbinil

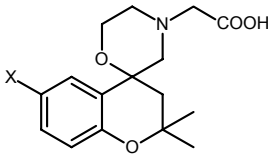


A

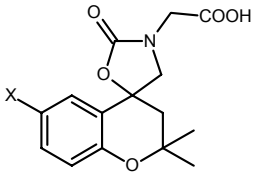
In particular, were synthesised compounds in which the spiroheterocycle is represented by a spiromorpholone- (**34a,b**), spiromorpholine- (**35a,b**) or a spirooxazolidinone (**36a,b**) ring respectively. The nitrogen atom of the spiroheterocycle core is substituted with an acetic chain; these derivatives still present the gem-methyl group in 2-position of the benzopyran.



34a,b



35a,b



36a,b

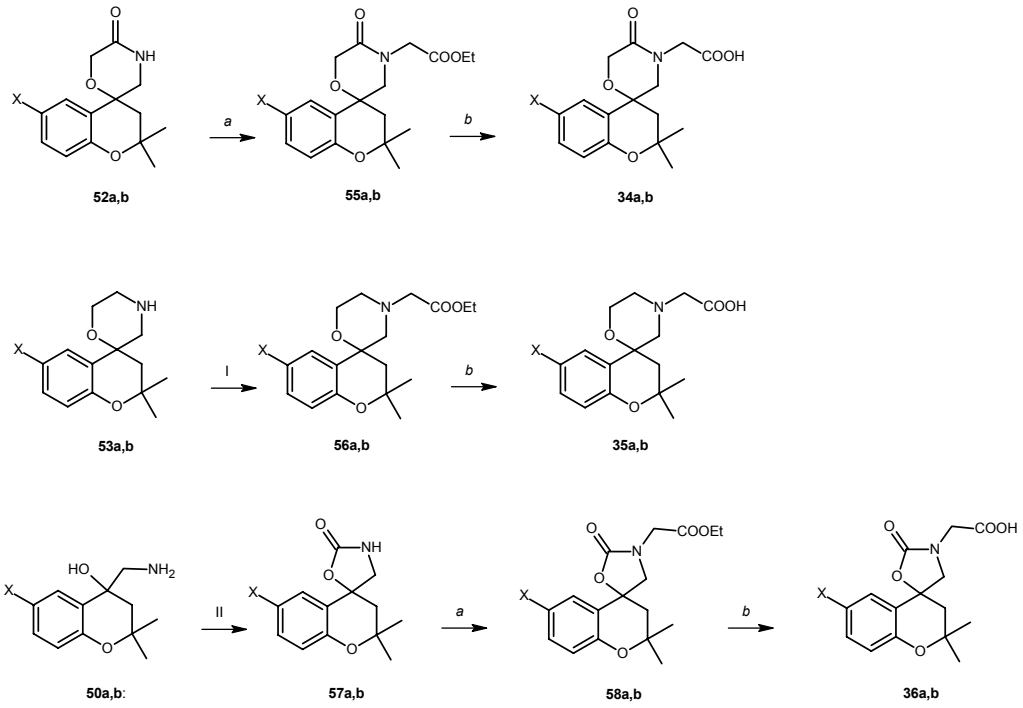
a: X= H

b: X=Br

Synthesis

Compounds **34a,b-36a,b** were synthesised following the synthetic procedure illustrated in Scheme 4.5 Spiromorpholones **52a,b** were obtained as previously described and their reaction with ethyl bromoacetate and NaH in DMF yielded compounds **55a,b**. Spiromorpholines **53a,b**, obtained as previously described, with ethylbromoacetate in the presence of K_2CO_3 yielded compounds **56a,b**. The subsequent cleavage of ester **55a,b** and **56a,b** with KOH in MeOH yielded compounds **34a,b** and **35a,b**. The aminoalcohols **50a,b** were obtained as previously described and their cyclization in the presence of *N-N*'-carbonyldiimidazole (CDI) yielded the corresponding spiro-oxazolidinone derivatives **57a,b**. Their reaction with ethyl bromoacetate and NaH in DMF yielded **58a,b** and their saponification with methanolic KOH yielded **36a,b**.

Scheme 4.5[a]



[a]Reagent and Conditions: (*a*) NaH, ethylbromoacetate, DMF, N_2; (*b*) KOH, MeOH; (I) K_2CO_3, ethylbromoacetate, CH_3CN; (II) CDI, THF.

Enzymatic Assays.

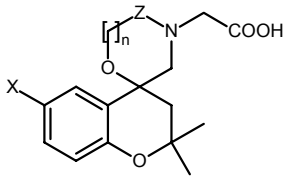
Synthesised compounds were assayed *in vitro* for their ability to inhibit ALR1 and ALR2. Rat lens, in which ALR2 is the predominant enzyme, and kidney, where ALR1 shows the highest concentration, were used for the isolation of ALR2 and ALR1, respectively. The activity of the two test enzymes was determined spectrophotometrically by monitoring the change in absorbance at 340 nm, which is due to the oxidation of NADPH catalyzed by ALR2 and ALR1. In order to evaluate the selectivity towards aldose reductase, synthesised compounds were also assayed for the inhibitory activity against sorbitol dehydrogenase (SDH).

Enzymatic Inhibition

The inhibitory activity of the new synthesised compounds against ALR2 and ALR1 was assayed by adding 0.1 mL of the inhibitor solution to the reaction mixture described above (see exp section). The inhibitory effect of the new derivatives was routinely estimated at a concentration of 10^{-4} M. The determination of the IC_{50} values was performed by linear regression analysis of the log-dose response curve, which was generated using at least four concentrations of the inhibitor causing an inhibition between 20% and 80%, with two replicates at each concentration. The 95% confidence limits (95% CL)

were calculated from *t* values for *n*-2, where *n* is the total number of determinations. Results are reported in Table 4.5, together with the IC_{50} values of the two well known ARL2 inhibitors sorbinil and tolrestat.

Table 4.5 ALR2 inhibition data of synthesised compounds and reference drugs.



34a,b-36a,b

Compd	X	Z	n	IC_{50} μM [a]
sorbinil				0.65
tolrestat				0.05
34a	H	CO	1	2.0
34b	Br	CO	1	n.a. [b]
35a	H	CH_2	1	n.a. [b]
35b	Br	CH_2	1	4.17
36a	H	CO	0	42.3
36b	Br	CO	0	17.3

[a] IC_{50} (95% CL) values represent the concentration required to produce 50% enzyme inhibition.
[b] n.a.: not active. Inhibition occurred at a concentration higher than 100 μM.

Discussion

The firstly synthesised compounds **34a,b-36a,b,** presenting the gem-dimethyl group in 2-position of the chromanic scaffold, showed a quiet controversial inhibitory behaviour, in particular reducing the carbonyl group of the spiromorpholic ring led to a loss of inhibitory activity as regards compound devoid of substituent in the C6 of the benzopyran nucleus (**34a** vs **35a**), while for the C6 brominated compounds was observed an opposite result (**34b** vs **35b**). As regards the introduction of a spirooxazolidinone moiety instead of a spiromorpholone one, also in this case was observed a reduction of the inhibitory activity for the C6-unsubsituted compound **36a**, while this kind of structural modification ameliorates the activity for the C6-brominated compound (**36b**).

Modification in 2-position: replacement of a methyl with a phenyl

Furthermore, my work kept on with the synthesis of new derivatives, in order to evaluate the importance of the steric hindrance in 2-position for the interaction with the active site of the enzyme. In particular, the chemical manipulation in this position concerned the replacement of a methyl group with an aromatic moiety, aiming to increase the possible interaction with the binding site of the enzyme. At present, it is well known the existence of an additional hydrophobic pocket of the enzyme. As revealed from X-ray analysis,[23] this novel subpocket is able to forms via the Trp 20 indole moiety a tight face-to-face π-π stacking

and the possibility to have the use of new structure of these possessing in 2-position a lipophilic group able to interact with this pocket might increase the activity of the new synthesised compounds.

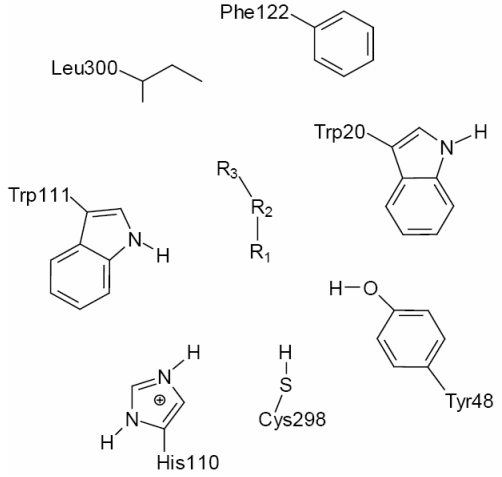
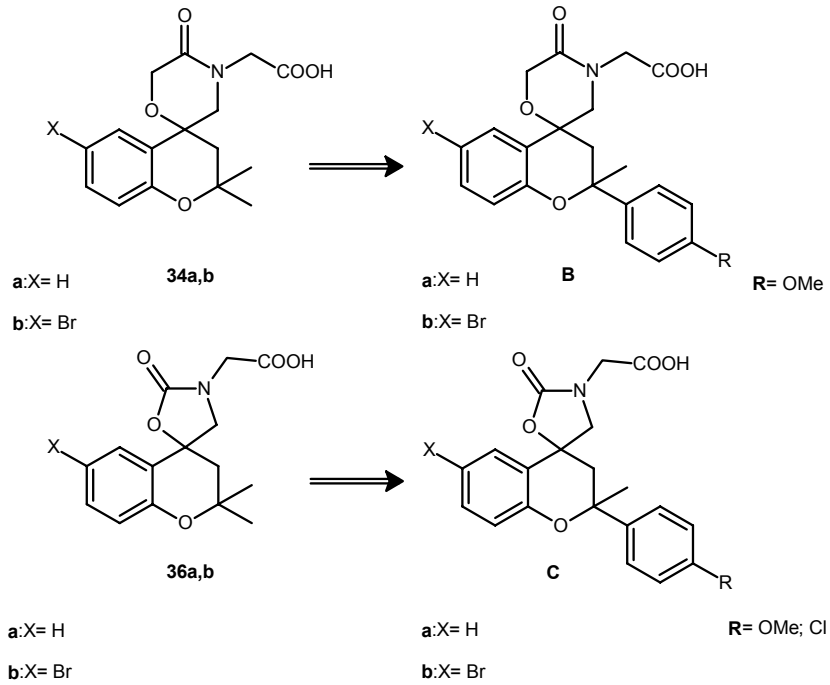


Figure 4.9 Interaction between generic AR inhibitors and the active site of the enzyme. R_1: acidic group for the interaction with the anionic site; R_2: lipophilic group for interaction with the hydrophobic pocket; R_3: additional lipophilic group for the interaction with the subpocket.

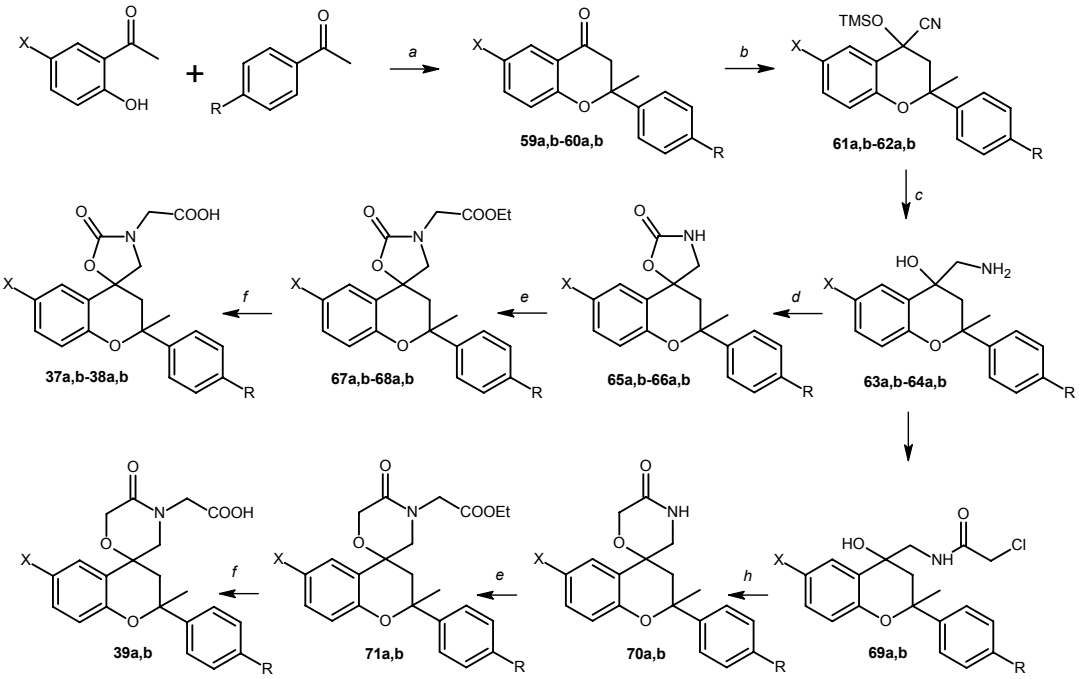
On this base, were synthesised compounds of type **B** and **C**, structurally related to **34a,b** and **36a,b**, in which one of the methyl of the gem-methyl was replaced by a *p*-methoxy or a *p*-chlorophenyl group.



Synthesis

Chromanones **59a,b** and **60a,b** were obtained from the appropriate 2-hydroxyacetophenone and 4-methoxy or 4-chloro acetophenone respectively, and subjected to a nucleophilic addition with TMSC in the presence of ZnI_2. Aminoalcohols **63a,b**, **64a,b** directly obtained by reduction of corresponding trimethylsilylcyanohydrines, were cyclizated in the presence of CDI to afford **65a,b**, **66a,b**. Reaction of **65a,b**, **66a,b** with ethyl bromoacetate in the presence of *n*-BuLi led to the corresponding *N*-ethylacetate derivatives. The cleavage of the ester with KOH in MeOH yielded **37a,b-39a,b**. Chloroacetamides **69a,b** were obtained from aminoalcohols **63a,b** and chloacetylchloride in heterogeneous phase and their base-catalized (*t*ButOK) cyclization yielded the spiromorpholone derivatives **70a,b**. The corresponding *N*-ethylacetate derivatives **71a,b** were obtained by reaction with ethyl bromoacetate and NaH in DMF and the cleavage of the esthers with KOH in MeOH give compounds **39a,b**.

Scheme 4.6[a]

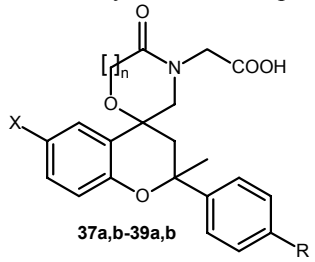


Compd	X	R
37a	H	OMe
37b	Br	OMe
38a	H	Cl
38b	Br	Cl
39a	H	OMe
39b	Br	OMe

[a] Reagent and conditions: (*a*) pyrrolidine, CH_3CN; (*b*) TMSCN, CH_2Cl_2,; (*c*) $LiAlH_4$, THF; (*d*) CDI, THF; (*e*) *n*-BuLi, ethylbromoacetate, THF, -78ºC, N_2; (*f*) KOH, MeOH; (*g*) chloroacetylchlori- de, NaOH, CH_2Cl_2/H_2O; (*h*) *t*ButOK, toluene; (*i*) NaH, ethylbromoacetate, DMF; (*l*) KOH, MeOH.

Enzymatic Assays.
Synthesised compounds were assayed *in vitro* for their ability to inhibit ALR1 and ALR2 and SDH. Enzymatic inhibition was evaluated as previously reported.

Tab. 4.5 ALR2 inhibition data of synthesised compounds and reference drugs.



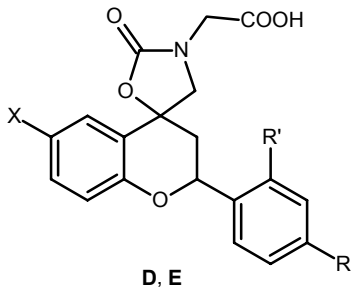
37a,b-39a,b

Compd	X	n	R	IC_{50} μM [a]
sorbinil				0.65
tolrestat				0.05
37a	H	0	OMe	43.8
37b	Br	0	OMe	1.60
38a	H	0	Cl	6.11
38b	Br	0	Cl	6.30
39a	H	1	OMe	n.a. [b]/48.42
39b	Br	1	OMe	4.97

[a] IC_{50} (95% CL) values represent the concentration required to produce 50% enzyme inhibition.
[b] n.a.: not active. Inhibition occurred at a concentration higher than 100 μM.

Discussion
The insertion of a lipophilic group (i. e. a phenyl substituted ring) instead of a gem-dimethyl substituent in 2-position, was generally favourable for the AR inhibition, with the only exception of **39a** in which this kind of subsituent was detrimental for the activity. In particular, **37b** showed a great improvement of the activity respect its analogous **36b** (IC_{50} =17.3 vs 1.60) and for the spiromorpholone derivative **39b** a satisfactory inhibitory behaviour was observed.

Modification in 2-position: displacement of the methyl group
The methyl group of compounds **37a,b-38a,b** was also removed, in order to evaluated if a reduced steric hindrance may improve the aminoacidic interaction of the new derivatives with the hydrophobic pocket, and were synthesised derivatives of type **D**, in which the substituents at 2-position are represented by an hydrogen atom and a *p*-methoxy or a *p*-chlorophenyl group. Moreover, was inserted a 2,4-di-halo-substituted phenyl ring in 2-position (**E**) accordingly with the observation that this kind of substituents could improve the activity, as observed for the **Ponalrestat**.



D, **E**

a::X= H;

b :X= Br

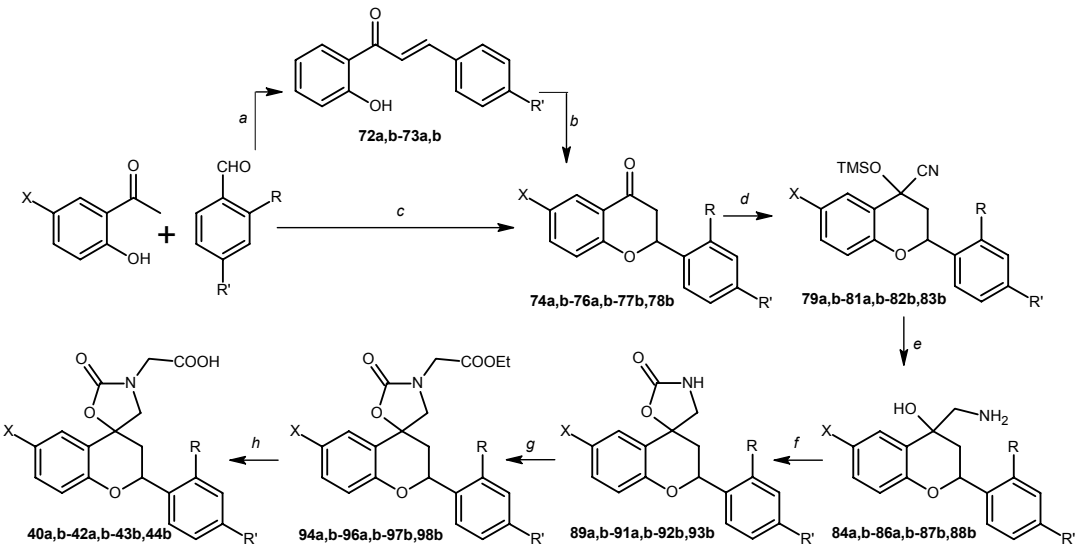
R= Cl; F; Br

R'= Cl; F

Synthesis

Compounds **40a,b-41a,b** were synthesised starting from the appropriate 2-hydroxyacetophenone and the appropriate benzaldehyde. The α-β insaturated compounds obtained were directly cyclizated with glacial AcOH to yield the corresponding chromanones **72a,b-73a,b**[24]. Chromanones **72a,b**, **73b** and **74b** were directly obtained from the appropriate 2-hydroxyacetophenone and the appropriate benzaldehyde in the presence of KOH. Compounds **74a,b-76a,b**, **77b** and **78b** were subjected to a nucleophilic addition with TMSC in the presence of ZnI_2. Aminoalcohols **84a,b-86a,b**,**87b** and **88b** directly obtained by reduction of corresponding trimethylsilylcyanohydrines, were cyclizated in the presence of CDI to afford **89a,b-91a,b**, **92b** and **93b**. Spirooxalidinones **89a,b-91a,b**, **92b** and **93b**, reacted with ethyl bromoacetate in the presence of *n*-BuLi to give the corresponding *N*-ethylacetate derivatives. The cleavage of the ester with KOH in MeOH yielded **40a,b-42a,b**, **43b** and **44b**.

Scheme 4.7[a]

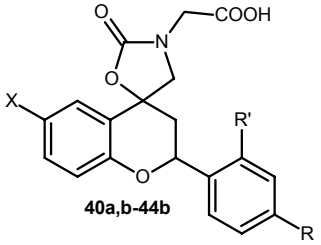


Compd	X	R	R'
40a	H	H	OMe
40b	Br	H	OMe
41a	H	H	Cl
41b	Br	H	Cl
42a	H	Cl	Cl
42b	Br	Cl	Cl
43b	Br	F	F
44b	Br	F	Br

[a] Reagent and conditions: (*a*) pyrrolidine, CH_3CN; (*b*) KOH, MeOH; (*c*) AcOH; (*d*) TMSCN, CH_2Cl_2; (*e*) $LiAlH_4$, THF; (*f*) CDI, THF; (*g*) *n*-BuLi, ethylbromoacetate, THF, -78 ºC, N_2; (*h*) KOH, MeOH.

Enzymatic Assays.
Synthesised compounds were assayed *in vitro* for their ability to inhibit ALR1 and ALR2 and SDH. Enzymatic inhibition was evaluated as previously reported.

Table 4.6 ALR2 inhibition data of the synthesised compounds and the reference drugs.



40a,b-44b

Compd	X	R'	R	IC_{50} µM [a]
sorbinil				0.65
tolrestat				0.05
40a	H	H	OMe	2.25
40b	Br	H	OMe	0.58
41a	H	H	Cl	5.69
41b	Br	H	Cl	8.25
42a	H	Cl	Cl	3.43
42b	Br	Cl	Cl	13.7
43b	Br	F	F	1.97
44b	Br	F	Br	6.19

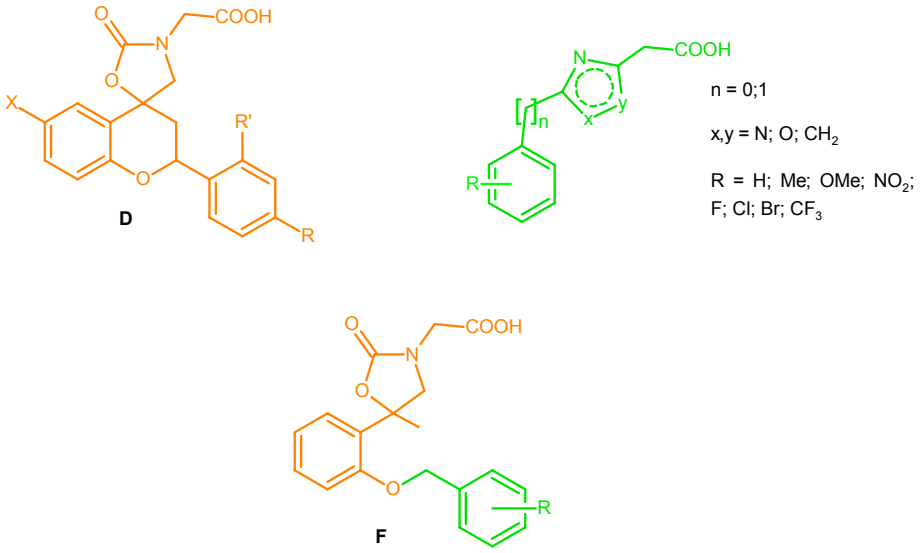
[a] IC_{50} (95% CL) values represent the concentration required to produce 50% enzyme inhibition.

Results and Discussion
Displacement of the methyl group from compounds **37a,b-38a,b** resulted in a further improvement of the inhibitory activity, with IC_{50} ranging from 0.58 µM (**40a**) and 13.7 µM (**42b**). Data reported in Table 2.6 indicate that both the mono- and the di-halo-substitution of the phenyl ring did not influence significantly the activity. The *para*-methoxy-subsituted derivative **40b** showed the better result with an IC_{50} value comparable with that of the reference drug sorbinil.

Experimental data of this limited series of synthesised compounds indicate some consideration: 1) the spirooxazolidinonic moiety is preferred to a spiromorpholone one; 2) a phenylic pendant at 2-position of the benzopyran nucleus is a favourable features for the inhibitory activity; 3) insertion of a bromine atom at the C6 of the benzopyran nucleus is generally well-accepted.

Further modification: replacement of the benzopyran scaffold

Finally, starting from the observation that some compounds described in literature[25] endowed of structural flexibility showed IC_{50} values in the micromolar range, were synthesised derivatives of type **F**, in which the spiro-oxazolidinone moiety is still present and the benzopyran scaffold is replaced by a benzyloxy pendant.

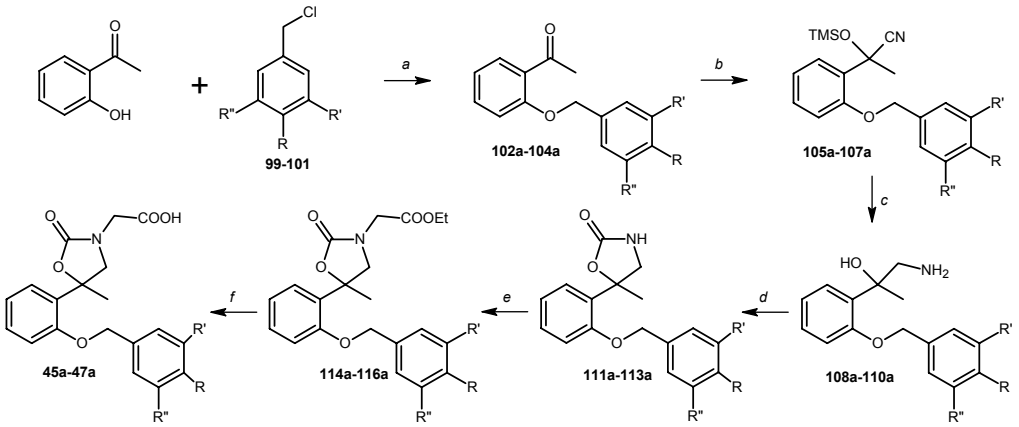


Compounds **F** present a 4-methoxy-subsituted benzyl ring (**45a**), 3,4-dimethoxy (**46a**) or 3,4,5-trimethoxy-subsituted benzyl ring (**47a**).

Synthesis

Compounds **45a-47a** were synthesised starting from 2-hydroxyacetophenone and the appropriate benzylchorides. Benzyloxyphenylethanones **102a-104a** were subject to a nucleophilic addition with TMSCN in the presence of ZnI_2 to give the corresponding trimethylsilylcyanohydrins **105a-107a**. Aminoalcohols **108a-110a**, directly obtained by reduction of **105a-107a**, were cyclizated in the presence of CDI. Spirooxalidinones **111a-113a** reacted with ethyl bromoacetate in the presence of *n*-BuLi to give the corresponding *N*-ethylacetate derivatives. The cleavage of the ester with KOH in MeOH yielded **45a-47a** (Scheme 2.8).

Scheme 4.8[a]

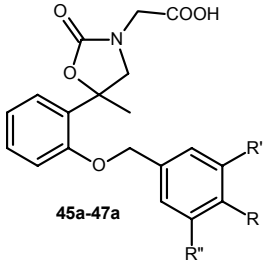


Compd	R	R'	R"
45a	H	H	OMe
46a	H	OMe	OMe
47a	OMe	OMe	OMe

[a] Reagent and conditions: (*a*) pyrrolidine, CH_3CN; (*b*) TMSCN, CH_2Cl_2,; (*c*) $LiAlH_4$, THF; (*d*) CDI, THF; (*e*) *n*-BuLi, ethylbromoacetate, THF, -78 ºC, N_2; (*f*) KOH, MeOH;

Enzymatic Assays.
Synthesised compounds were assayed *in vitro* for their ability to inhibit ALR1 and ALR2 and SDH. Enzymatic inhibition was evaluated as previously reported.

Table 4.7 ALR2 inhibition data of synthesised compounds and the reference drugs.



45a-47a

Compd	R	R'	R"	IC_{50} µM [a]
sorbinil				0.65
tolrestat				0.05
45a	H	H	OMe	4.13
46a	H	OMe	OMe	n.t.
47a	OMe	OMe	OMe	3.19

[a] IC_{50} (95% CL) values represent the concentration required to produce 50% enzyme inhibition.

Results
Data reported in Table 4.7. seems to indicate that also the replacement of the benzopyran scaffold with a more flexible nucleus is a well tolerated chemical manipulation that could led to new compounds endowed of AR inhibitory activity. To establish if this nucleus enhance the affinity and selectivity of these compounds towards AR, other analogous variously substituted might be synthesised.

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5 EXPERIMENTAL *SECTION*

5.1 Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. NMR spectra were obtained with a Varian Gemini 200 MHz spectrometer. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane and referenced from solvent references. The elemental compositions of the compounds agreed to within (0.4% of the calculated value. Chromatographic separation was performed on silica gel columns by flash (Kieselgel 40, 0.040-0.063 mm; Merck) or gravity column (Kieselgel 60, 0.063-0.200 mm; Merck) chromatography. Reactions were followed by thin-layer chromatography (TLC) on Merck aluminum silica gel (60 F_{254}) sheets that were visualized under a UV lamp. Evaporation was performed in vacuo (rotating evaporator). Sodium sulfate was always used as the drying agent. The microwave-assisted procedures were carried out with a CEM Discover LabMate Microwave. Commercially available chemicals were purchased from Sigma-Aldrich.

4′-(2-methoxybenzyl)-2,2-dimethyl-2,3-dihydro-52*H*-spiro-[chromene-4,2′ [1,4]oxazinan]-52-one 15a.

To a stirred solution of NaH (0.07 g, 3.00 mmol, 60% dispersion in mineral oil) in dry DMF (10 mL) was added **52a** (0.9 g, 4.00 mmol) under N2 atmosphere. After 30 min, the reaction mixture was cooled at 0 °C and 2-methoxybenzyl bromide (0.78 g, 5.00 mmol) was added. The reaction mixture was allowed to warm at 25 °C and stirred for 1 h before being quenched with water and extracted with EtOAc. The combined organic layers were dried, filtered, and concentrated under vacuum. The crude product was purified by flash column chromatography eluting Hexane/EtOAc (1/1) to give **15a** (0.44 g, 1.20 mmol, 30% yield) as an oil.

^{1}H-NMR ($CDCl_3$) δ: 1.15 (s, 3H, Me); 1.33 (s, 3H, Me); 1.77 (d, 1H, J = 14.5 Hz, CH_2); 2.25 (d, 1H, J = 14.5 Hz, CH_2); 3.11 (d, 1H, J = 12.6 Hz, CH_2); 3.79 (s, 3H, OMe); 3.82 (d, 1H, J = 12.6 Hz, CH_2); 4.26 (d, 1H, J = 17.4 Hz, CH_2); 4.38 (d, 1H, J = 17.4 Hz, CH_2); 4.48 (d, 1H, J = 14.2 Hz, CH2N); 4.94 (d, 1H, J = 14.2 Hz, CH_2N); 6.78-6.98 (m, 4H, Ar); 7.17-7.41 (m, 4H, Ar) ppm. ^{13}C NMR ($CDCl_3$) δ: 166.49, 157.79, 153.67, 131.04, 130.16, 129.42, 127.43, 123.97, 121.86, 120.86, 120.75, 118.15, 110.55, 74.26, 69.17, 63.72, 55.42, 53.56, 44.08, 39.61, 28.60, 26.27. MS m/z: 367 (M^+ 22%). Anal. ($C_{22}H_{25}NO_4$) C, H, N.

4′-(2-methoxybenzyl)-6-bromo-2,2-dimethyl-2,3-dihydro-52*H*spiro[chromene-4,2′-[1,4]oxazinan]-52-one 15b.

Compound **15b** was synthesized from **52b** (1.30 g, 4.00 mmol) and 2-methoxybenzyl bromide (0.78 g, 5.00 mmol) following the same procedure

described above for **15a**. The crude product was purified by flash column chromatography eluting with Hexane/EtOAc (1/1) to afford **15b** (0.37 g, 0.84 mmol, 21% yield): mp 48-50 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.11 (s, 3H, Me); 1.30 (s, 3H, Me); 1.74 (d, 1H, *J* = 14.6 Hz, CH_2); 2.22 (d, 1H, *J* = 14.6 Hz, CH_2); 3.10 (d, 1H, *J* = 12.8 Hz, CH_2); 3.72-3.84 (m, 1H, CH_2); 3.80 (s, 3H, OMe); 4.25 (d, 1H, *J* = 17.4 Hz, CH_2); 4.38 (d, 1H, *J* = 17.4 Hz, CH_2); 4.47 (d, 1H, *J* = 14.3 Hz, CH_2N); 4.93 (d, 1H, *J* = 14.3 Hz, CH_2N); 6.69 (d, 1H, *J* = 8.6 Hz, Ar); 6.85-7.00 (m, 2H, Ar); 7.24-7.35 (m, 3H, Ar); 7.50 (d, 1H, *J* = 2.4 Hz, Ar) ppm. ^{13}C NMR (CDCl3) δ: 166.41, 157.63, 152.33, 133.07, 131.00, 129.87, 129.56, 127.87, 123.46, 120.54, 120.19, 112.50, 110.21, 74.68, 68.99, 63.63, 55.78, 53.21, 44.33, 39.57, 28.80, 25.97. MS m/z: 446 (M^+ 38%). Anal. ($C_{22}H_{24}BrNO_4$) C, H, N.

4′-(3-methoxybenzyl)-2,2-dimethyl-2,3-dihydro-52*H*-spiro-[chromene-4,2′-[1,4]oxazinan]-52-one 16a.

Compound **16a** was synthesized from **52a** (0.9 g, 4.00 mmol) and 3-methoxybenzyl chloride (0.78 g, 5.00 mmol) following the same procedure described above for **15a**. The crude product was purified by flash column chromatography eluting Hexane/EtOAc (7/3) to give **16a** (0.79 g, 2.16 mmol, 54% yield) as an oil. ^{1}H-NMR ($CDCl_3$) δ: 1.14 (s, 3H, Me); 1.33 (s, 3H, Me); 1.76 (d, 1H, *J* = 14.5 Hz, CH_2); 2.26 (d, 1H, *J* = 14.5 Hz, CH_2); 3.08 (d, 1H, *J* = 12.4 Hz, CH_2); 3.79 (d, 1H, *J* = 12.4 Hz, CH_2); 3.80 (s, 3H, OMe); 4.24 (d, 1H, *J* = 14.3 Hz, CH_2N); 4.29 (d, 1H, *J* = 17.4 Hz, CH_2); 4.41 (d, 1H, *J* = 17.4 Hz, CH_2); 4.99 (d, 1H, *J* = 14.3 Hz, CH_2N); 6.79-6.94 (m, 5H, Ar); 7.17-7.30 (m, 2H, Ar); 7.37 (dd, 1H, *J* = 1.6, 7.9 Hz, Ar) ppm. ^{13}C NMR ($CDCl_3$) δ: 166.51, 160.03, 153.67, 131.48, 130.29, 129.89, 127.34, 121.66, 121.19, 120.80, 118.24, 114.43, 113.58, 74.25, 69.15, 63.69, 55.40, 55.20, 49.74, 39.69, 28.65, 26.21. MS m/z: 367 (M^+ 45%). Anal. ($C_{22}H_{25}NO_4$) C, H, N.

4′-(3-methoxybenzyl)-6-bromo-2,2-dimethyl-2,3-dihydro-52*H*spiro[chromene-4,22-[1,4]oxazinan]-52-one 16b.

Compound **16b** was synthesized from **52b** (1.30 g, 4.00 mmol) and 3-methoxybenzyl bromide (0.78 g, 5.00 mmol) following the same procedure described above for **15a**. The crude product was purified by flash column chromatography eluting with Hexane/EtOAc (7/3) to obtain **16b** (0.57 g, 1.28 mmol, 32% yield) as an oil. ^{1}H-NMR ($CDCl_3$) δ: 1.11 (s, 3H, Me); 1.31 (s, 3H, Me); 1.74 (d, 1H, *J* = 14.6 Hz, CH_2); 2.23 (d, 1H, *J* = 14.6 Hz, CH_2); 3.06 (d, 1H, 12.4 Hz, CH_2); 3.72-3.80 (m, 1H, CH_2); 3.80 (s, 3H, OMe); 4.23 (d, 1H, *J* = 14.2 Hz, CH_2N); 4.28 (d, 1H, *J* = 17.4 Hz, CH_2); 4.41 (d, 1H, *J* = 17.4 Hz, CH_2); 4.97 (d, 1H, *J* = 14.2 Hz, CH_2N); 6.69 (d, 1H, *J* = 8.8 Hz, Ar); 6.83-6.88 (m, 3H, Ar); 7.22-7.32 (m, 2H, Ar); 7.49 (d, 1H, *J* =2.4 Hz, Ar) ppm. ^{13}C NMR ($CDCl_3$) δ: 166.52, 160.12, 152.45, 133.26, 131.09, 130.41, 129.68, 127.64, 120.55, 120.23, 114.16, 113.88, 112.49, 74.50, 69.04, 63.53, 55.30, 55.21,

49.60, 39.81, 28.55, 25.14. MS m/z: 446 (M^+ 51%). Anal. ($C_{22}H_{24}BrNO_4$) C, H, N.

4'-(4-bromobenzyl)-2,2-dimethyl-2,3-dihydro-5'H-spiro[chromene-4,2'-[1,4] oxazinan]-5'-one 17a.

Compound **17a** was synthesized from **52a** (0.9 g, 4.00 mmol) and 4-bromobenzyl bromide (1.30 g, 5.00 mmol) following the same procedure described above for **15a**. The crude product was purified by trituration with Et_2O to give **17a** (0.63 g, 1.52 mmol, 38 % yield): mp 134-136 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.20 (s, 3H, Me); 1.33 (s, 3H, Me); 1.72 (d, 1H, *J* = 14.6 Hz, CH_2); 2.27 (d, 1H, *J* =14.6 Hz, CH_2); 3.04 (d, 1H, *J* = 12.4 Hz, CH2); 3.79 (d, 1H, *J* = 12.4 Hz, CH_2); 4.29-4.44 (m, 3H, CH_2, CH_2); 4.87 (d, 1H, *J* = 14.4 Hz, CH_2); 6.80-6.95 (m, 2H, Ar); 7.16-7.22 (m, 3H, Ar); 7.35(dd, 1H *J* = 1.5, 7.9 Hz, Ar); 7.48 (d, 2H, *J* = 8.4 Hz, Ar) ppm. ^{13}C-NMR ($CDCl_3$) δ: 166.69, 153.78, 135.16, 132.03, 130.55, 130.30, 127.20, 122.19, 121.74, 120.79, 118.35, 74.21, 69.23, 63.67, 55.73, 49.47, 40.16, 29.13, 26.04. MS (m/z): 416 (M^+ 46%). Anal. ($C_{21}H_{22}BrNO_3$) C, H, N.

4'-(4-bromobenzyl)-6-bromo-2,2-dimethyl-2,3-dihydro-5'H-spiro[chromene-4,2'-[1,4]oxazinan]-5'-one 17b.

Compound **17b** was synthesized from **52b** (1.30 g, 4.00 mmol) and 4-bromobenzyl chloride (1.25 g, 5.00 mmol) following the same procedure described above for **15a**. The crude product was purified by trituration with Et_2O/Hex to give **17b** (0.51 g, 1.04 mmol, 26 % yield): mp 83-85 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.18 (s, 3H, Me); 1.31 (s, 3H, Me); 1.69 (d, 1H, *J* = 14.6 Hz, CH_2); 2.24 (d, 1H, *J* = 14.6 Hz, CH_2); 3.04 (d, 1H, *J* =12.4 Hz, CH_2); 3.74 (d, 1H, *J* = 12.4 Hz, CH_2); 4.23-4.49 (m, 3H, CH_2); 4.83 (d, 1H, *J* = 14.5 Hz, CH_2); 6.70 (d,1H, *J* = 8.8 Hz, Ar); 7.16-7.34 (m, 4H, Ar); 7.45-7.50 (m, 2H, Ar) ppm. ^{13}CNMR ($CDCl_3$) δ: 166.29, 152.88, 133.54, 133.26, 132.31, 130.23, 127.58, 125.62, 123.41, 120.50, 112.90, 74.11, 69.30, 63.54, 55.65, 49.40, 39.89, 29.05, 26.12. MS (m/z): 495 (M^+ 36%). Anal. ($C_{21}H_{21}Br_2NO_3$) C, H, N.

4′-(2-methoxybenzyl)-2,2-dimethyl-2,3-dihydrospiro[chromene-4,2′-[1,4] oxazinane] 18a.

To a solution of **53a** (0.13 g, 0.57 mmol) in MeCN (5 mL) was added K_2CO_3 (0.09 g, **0.64** mmol) and 2-methoxy-benzylchloride (0.09 g, 0.57 mmol). The resulting mixture was refluxed for 12 h, then, after cooling, was filtered and the solvent evaporated. The crude product was transformed into the hydrochloride salt and crystallized from *i*-PrOH to give **18a** (0.29 g, 0.76 mmol, 37% yield): mp 185-187 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.27 (s, 3H, Me); 1.36 (s, 3H, Me); 2.13 (d, 1H, *J* = 14.6 Hz, CH_2); 2.33-2.59 (m, 4H, CH_2, CH_2); 2.30 (d, 1H, *J* = 12.7 Hz, CH_2); 2.70-2.75 (m, 1H, CH_2); 3.53 (d, 1H, *J* = 12.7 Hz, CH_2); 3.72-4.01 (m, 2H, CH_2); 3.79 (s, 3H, OMe); 6.76-6.95 (m, 4H, Ar); 7.12-7.26 (m, 3H,

Ar); 7.62 (d, 1H, *J* = 7.7 Hz, Ar) ppm. ^{13}C NMR ($CDCl_3$) δ: 158.28, 153.98, 134.21, 132.15, 130.60, 126.85, 121.68, 121.41, 120.73, 118.64, 115.69, 111.14, 74.61, 69.99, 61.70, 57.88, 55.76, 55.00, 51.54, 39.45, 28.47, 26.27. MS (m/z): 353 (M^+ 58%). Anal. ($C_{22}H_{27}NO_3$·HCl) C, H, N.

4′-(2-methoxybenzyl)-6-bromo-2,2-dimethyl-2,3-dihydrospiro[chromene-4,2′ [1,4]oxazinane] 18b.
Compound **18b** was synthesized from **53b** (0.18 g, 0.57 mmol) and 2-methoxybenzylchloride (0.09 g, 0.57 mmol) following the same procedure described above for the preparation of **18a**. The crude residue was transformed into the hydrochloride salt and crystallized from *i*-PrOH to yield **18b** (0.1 g, 0.21 mmol, 26% yield): mp 158-160 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.33 (s, 3H, Me); 1.36 (s, 3H, Me); 2.61(d, 1H, *J* = 15.0 Hz, CH_2); 2.85 (d, 1H, *J* = 15.0 Hz, CH_2); 2.95-3.15 (m, 2H, CH_2); 3.58-4.03 (m, 4H, CH_2); 3.85 (s, 3H, OMe); 4.35-4.60 (m, 2H, CH_2); 6.72 (d, 1H, *J* = 8.8 Hz, Ar); 6.94 (d, 1H, *J* = 8.2 Hz, Ar); 7.03-7.11 (m, 1H, Ar); 7.29-7.47(m, 3H, Ar); 7.86-7.90 (m, 1H, Ar). ^{13}C NMR ($CDCl_3$) δ: 157.95, 152.81, 132.15, 131.13, 130.42, 128.31, 126.94, 125.98, 120.37, 119.60, 112.36, 110.61, 75.21, 70.06, 63.47, 61.32, 56.40, 55.60, 53.69, 40.74, 29.13, 27.00. MS (m/z): 432 (M^+, 31%). Anal. ($C_{22}H_{26}BrNO_3$·HCl) C, H, N.

4′-(3-methoxybenzyl)-2,2-dimethyl-2,3-dihydrospiro[chromene-4,2′-[1,4] oxazinane] 19a.
Compound **19a** was synthesized from **53a** (0.08 g, 0.34 mmol) and 3-methoxybenzylbromide (0.07 g, 0.34 mmol) following the same procedure described above for the preparation of **18a**. The crude product was transformed to the hydrochloride salt and crystallized from *i*-PrOH to yield **19a** (0.3 g, 0.83 mmol, 40% yield): mp 141-143 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.32 (s, 3H, Me); 1.35 (s, 3H, Me); 2.63 (d, 1H, *J* = 15.0 Hz, CH_2); 2.83 (d, 1H, *J* = 15.0 Hz, CH_2); 2.94-3.08 (m, 2H, CH_2); 3.65-3.99 (m, 4H, CH_2, CH_2); 3.84 (s, 3H, OMe); 4.55-4.69 (m, 2H, CH_2); 6.82 (d, 1H, *J* = 8.1 Hz, Ar); 6.90-6.98 (m, 2H, Ar); 7.14-7.35 (m, 4H, Ar); 7.43-7.56 (m, 1H, Ar). ^{13}C NMR ($CDCl_3$) δ: 160.19, 153.89, 130.67, 130.44, 128.93, 127.05, 123.32, 121.28, 120.79, 118.58, 116.58, 116.25, 74.57, 70.04, 61.85, 58.44, 57.82, 55.71, 52.58, 39.54, 28.23, 26.63. MS (m/z): 353 (M^+ 58%). Anal. ($C_{22}H_{27}NO_3$·HCl) C, H, N.

4′-(3-methoxybenzyl)-6-bromo-2,2-dimethyl-2,3-dihydrospiro[chromene-4,2′ [1,4]oxazinane] 19b.
Compound **19b** was synthesized from **53b** (0.13 g, 0.43 mmol) and 3-methoxybenzylbromide (0.07 g, 0.43 mmol) following the same procedure described above for the preparation of **18a** The crude residue was transformed to the hydrochloride salt to give **19b** (0.07 g, 0.14 mmol, 41% yield): mp 141-143 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.28 (s, 3H, Me); 1.33 (s, 3H, Me); 2.64-3.09 (m, 4H, CH_2); 3.66-3.99 (m, 4H, CH_2); 3.84 (s, 3H, OMe); 4.53-4.68 (m, 2H, CH_2);

6.70 (d, 1H, *J* = 8.8 Hz, Ar); 6.96 (dd, 1H, *J* = 1.5, 8.2 Hz, Ar); 7.16-7.36 (m, 3H, Ar); 7.45-7.48 (m, 2H, Ar) ppm ^{13}C NMR ($CDCl_3$) δ: 160.10, 153.21, 133.25, 131.35, 129.89, 129.75, 123.52, 121.23, 120.16, 114.36, 113.20, 112.79, 75.28, 69.73, 61.95, 58.30, 57.97, 55.46, 52.50, 39.49, 28.05, 26.88. MS (m/z): 432 (M^+ 31%). Anal. ($C_{22}H_{26}BrNO_3 \cdot HCl$) C, H, N.

4′-(4-bromobenzyl)-2,2-dimethyl-2,3-dihydrospiro[chromene-4,2′-[1,4] oxazinane] 20a.

Compound **20a** was synthesized from **53a** (0.15 g, 0.64 mmol) and 4-bromobenzylbromide (0.16 g, 0.64 mmol) following the same procedure described above for the preparation of **18a**. The crude product was transformed into the hydrochloride salt to give **20a** (0.08 g, 0.19 mmol, 30% yield): mp 182-184 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.33 (s, 6H, Me); 2.51 (d, 1H, *J* = 15.2 Hz, CH_2); 2.86 (d, 1H, *J* = 15.2 Hz, CH_2); 2.95-3.03 (m, 2H, CH_2); 3.65-3.98 (m, 4H, CH_2); 4.51-4.65 (m, 2H, CH_2); 6.80-6.98 (m, 2H, Ar); 7.19-7.35 (m, 2H, Ar); 7.54 (d, 2H, *J* = 8.4 Hz, Ar); 7.63 (d, 2H, *J* = 8.4 Hz, Ar) ppm. ^{13}C NMR ($CDCl_3$) δ: 153.93, 148.23, 147.19, 146.04, 133.15, 132.71, 130.76, 127.00, 120.82, 118.69, 74.54, 70.09, 61.12, 58.20, 57.80, 52.16, 39.56, 28.49, 26.36. MS (m/z): 402 (M^+ 34%). Anal. ($C_{21}H_{24}BrNO_2 \cdot HCl$) C, H, N.

4′-(4-bromobenzyl)-6-bromo-2,2-dimethyl-2,3-dihydrospiro[chromene-4,2′ [1,4]oxazinane] 20b.

Compound 20b was synthesized from **53b** (0.21 g, 0.76 mmol) and 4-bromobenzylbromide (0.19 g, 0.76 mmol) following the same procedure described above for the preparation of **18a**. The crude residue was transformed into the hydrochloride salt and crystallized from *i*PrOH to give **20b** (0.5 g, 0.93 mmol, 45% yield): mp 168-170 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.30 (s, 3H, Me); 1.32 (s, 3H, Me); 2.56 (d, 1H, *J* = 15.2 Hz, CH_2); 2.80 (d, 1H, *J* = 15.2 Hz, CH_2); 2.89-3.04 (m, 2H, CH_2); 3.66-3.99 (m, 4H, CH_2); 4.50-4.67 (m, 2H, CH_2); 6.70 (d, 1H, *J* = 8.8 Hz, Ar); 7.31 (dd, 1H, *J* = 2.3, 8.8 Hz, Ar); 7.46 (d, 1H, *J* = 2.3 Hz, Ar); 7.56 (d, 2H, *J* = 8.4 Hz, Ar); 7.67 (d, 2H, *J* = 8.4 Hz, Ar). ^{13}C NMR ($CDCl_3$) δ: 153.14, 133.70, 133.22, 132.79, 129.91, 126.45, 125.21, 123.32, 120.66, 112.76, 75.16, 69.99, 61.19, 58.44, 57.88, 52.67, 39.40, 28.33, 26.49. MS (m/z): 481 (M^+ 57%). Anal. ($C_{21}H_{23}Br_2NO_2 \cdot HCl$) C, H, N.

4'-benzyl-2,2-dimethyl-2,3-dihydro-5'*H*-spiro[chromene-4,2'-[1,4]oxazinane]-5'-thione 21a.

To a stirred solution of **6a** (0.1 g, 0.3 mmol) in chlorobenzene Lawesson's Reagent (0.17 mmol) was added. The reaction mixture was refluxed for 2h and then the solvent was evaporated. The crude residue was crystallized from EtOH to give **21a** (0.06 g, 0.17 mmol, yield 57%): mp 154-158 °C. ^{1}H-NMR ($CDCl_3$) δ:1.04 (s, 3H, Me); 1.30 (s, 3H, Me); 1.59 (d, 1H, *J* = 14.5 Hz, CH_2); 2.15 (d, 1H, *J* = 14.5 Hz, CH_2); 3.25 (d, 1H, *J* = 13.4 Hz, CH_2); 3.78 (d, 1H, *J* = 13.4

Hz, CH_2); 4.59-4.92 (m, 3H, CH_2); 5.99 (d, 1H, *J* = 13.9 Hz, CH_2); 6.79 (d, 1H, *J* = 8 Hz, Ar); 6.88-6.96 (m, 1H, Ar); 7.18-7.43 (m, 7H, Ar) ppm. ^{13}C NMR ($CDCl_3$) δ: 195.76, 153.80, 134.74, 130.70, 129.21, 128.84, 127.55, 121.36, 121.16, 118.46, 74.36, 70.32, 69.36, 56.79, 56.72, 39.60, 28.33, 26.46. Anal. ($C_{21}H_{23}NO_2S$) C, H, N.

4'-benzyl-6-bromo-2,2-dimethyl-2,3-dihydro-5'H-spiro[chromene-4,2'-[1,4]oxazinane]-5'-thione 21b.
Compound **21b** was synthesized from **6b** following the same procedure described above for **21a**. The crude product was purified by flash column chromatography eluted with Hex/EtOAc to give **21b** (yield 61%) mp170-172 C. ^{1}H-NMR ($CDCl_3$) δ: 1.00 (s, 3H, Me); 1.28 (s, 3H, Me); 1.56 (d, 1H, *J* = 14.5 Hz, CH_2); 2.12 (d, 1H, *J* = 14.5 Hz, CH_2); 3.24 (d, 1H, *J* = 13.5 Hz, CH_2); 3.74 (d, 1H, *J* = 13.5 Hz, CH_2); 4.58–4.92 (m, 3H, CH_2); 5.98 (d, 1H, *J* = 14.1 Hz, CH_2); 6.68 (d, 1H, *J* = 8.8 Hz, Ar); 7.26–7.46 (m, 7H, Ar) ppm. Anal. ($C_{21}H_{22}BrNO_2S$) C, H, N.

4'-(4-methylbenzyl)-2,2-dimethyl-2,3-dihydro-5'H-spiro[chromene-4,2'-[1,4]oxazinane]-5'-thione 22a.
Compound **22a** was synthesized from **7a** (0.4 g, 1.14 mmol) and Lawesson's Reagent (0.28 g, 0.68mmol) following the same procedure described above for **21a.** The crude product was purified by flash column chromatography eluted with Hex/EtOAc (95/5) to give **22a** (0.18 g, 0.49 mmol, yield 43%). ^{1}H-NMR ($CDCl_3$) δ:1.07 (s, 3H, Me); 1.29 (s, 3H, Me); 1.57 (d, 1H, J = 14.5 Hz, CH_2); 2.14 (d, 1H, J = 14.5 Hz, CH_2); 2.38 (s, 3H, Me); 3.21 (d, 1H, J = 13.5 Hz, CH_2); 3.74 (d, 1H, J = 13.5 Hz, CH_2); 4.63 (d, 1H, *J* = 13.9 Hz, CH_2); 4.69 (d, 1H, *J* = 18.9 Hz, CH_2); 4.84 (d, 1H, *J* = 18.9 Hz, CH_2); 5.87 (d, 1H, *J* = 13.9 Hz, CH_2); 6.79 (d, 1H, *J* = 8.2 Hz, Ar); 6.87-6.94 (m, 1H, Ar); 7.11–7.32 (m, 6H, Ar) ppm. ^{13}C NMR ($CDCl_3$): *δ* 195.61, 153.82, 138.66, 131.63, 130.65, 129.79, 127.53, 121.45, 121.12, 118.46, 74.36, 70.32, 69.37, 56.77, 56.54, 39.71, 28.55, 26.24, 21.51. Anal. ($C_{22}H_{25}NO_2S$) C, H, N.

4'-(4-bromobenzyl)-2,2-dimethyl-2,3-dihydro-5'H-spiro[chromene-4,2'-[1,4]oxazinane]-5'-thione 23a.
Compound **23a** was synthesized from **9a** (0.22 g, 0.53 mmol) and Lawesson's Reagent (0.26 g, 0.63 mmol). The reaction mixture was refluxed overnight. The solvent was evaporated under vaccum and the crude product was purified by trituration with CH2Cl2, to give **23a** (0.07 g, 0.16 mmol, yield 30%).
^{1}H-NMR ($CDCl_3$) δ : 1.16 (s, 3H, Me); 1.32 (s, 3H, Me); 1.60 (d, 1H, J = 14.6 Hz, CH_2); 2.18 (d, 1H, J = 14.6 Hz, CH_2); 3.20 (d, 1H, J = 13.4 Hz, CH_2); 3.77 (d, 1H, J = 13.4 Hz, CH_2); 4.70 (d, 1H, *J* = 19.1 Hz, CH_2); 4.75 (d, 1H, *J* = 14.2 Hz, CH_2); 4.85 (d, 1H, *J* = 19.1 Hz, CH_2); 5.76 (d, 1H, *J* = 14.2 Hz, CH_2); 6.82 (dd, 1H, *J* = 1.1, 8.2 Hz, Ar); 6.88-6.96 (m, 1H, Ar); 7.18–7.32 (m, 4H, Ar);

7.49 (d, 2H, *J* = 8.4 Hz, Ar) ppm. ^{13}C NMR ($CDCl_3$): δ 196.19, 153.82, 133.72, 132.30, 130.79, 127.44, 122.87, 121.25, 121.18, 118.55, 74.33, 70.28, 69.37, 57.23, 56.19, 39.84, 28.93, 26.02. Anal. ($C_{21}H_{22}BrNO_2S$) C, H, N.

2,2-dimethyl-4'-(phenylcarbonyl)-2,3-dihydro-5'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-5'-one 24a.
A solution of **52a** (0.2 g, 0.8 mmol) in dry THF was added dropwise, at -78 ˚C, under N_2 atmosphere to a solution of *n*-BuLi (0.850mL, 1.6 M in Hexane). The reaction mixtures were stirred for 1h and then benzoyl chloride (0.11 g, 0.8 mmol) was added dropwise at -78 ˚C and the resulting mixtures were allowed to warm to rt and stirred overnight. The mixtures were quenched with NH_4Cl_{sat} and then the solvent was evaporated. The residues were extracted with EtOAc, the combined organic layers were dried and concentrated under vacuum. The crude product was purified by trituration with Et2O to give **24a** (0.08 g, 0.24 mmol, yield 30%): mp 151-155 ˚C. ^{1}H-NMR ($CDCl_3$) δ: 1.43 (s, 3H, Me); 1.47 (s, 3H, Me); 2.11 (d, 1H, *J* = 14.5 Hz, CH_2); 2.43 (d, 1H, *J* = 14.5 Hz, CH_2); 4.01 (d, 1H, *J* = 13.7 Hz, CH_2); 4.24 (d, 1H, *J* = 13.7 Hz, CH_2); 4.35 (d, 1H, *J* = 17.7 Hz, CH_2); 4.47 (d, 1H, *J* = 17.7 Hz, CH_2); 6.87-7.03 (m, 2H, Ar); 7.23-7.32 (m, 1H, Ar); 7.41-7.57 (m, 5H, Ar); 7.64-7.69 (m, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$) δ: 172.56, 170.01, 153.71, 135.16, 132.70, 130.70, 128.68, 128.55, 127.09, 122.38, 121.30, 118.66, 74.51, 70.43, 64.73, 53.28, 41.12, 29.81, 25.97. Anal. ($C_{21}H_{21}NO_4$) C, H, N.

6-bromo-2,2-dimethyl-4'-(phenylcarbonyl)-2,3-dihydro-5'H-spiro[chromene-4,2'-[1,4]oxazinan]-5'-one 24b.
Compound **24b** was synthesized from **52b** (0.2 g, 0.6 mmol) following the same procedure described above for **24a**. The crude product was purified by crystallization from EtOH to give **24b** (0.05 g, 0.12 mmol, yield 20%). mp180-185 ˚C. ^{1}H-NMR ($CDCl_3$) δ: 1.40 (s, 3H, Me); 1.46 (s, 3H, Me); 2.09 (d, 1H, *J* = 14.7 Hz, CH_2); 2.39 (d, 1H, *J* = 14.7 Hz, CH_2); 4.02 (d, 1H, *J* = 13.7 Hz, CH_2); 4.16 (d, 1H, *J* = 13.7 Hz, CH_2); 4.33 (d, 1H, *J* = 17.7 Hz, CH_2); 4.48 (d, 1H, *J* = 17.7 Hz, CH_2); 6.78 (d, 1H, *J* = 8.7 Hz, Ar); 7.36 (dd, 1H, *J* = 2.4, 8.7; Ar); 7.42-7.69 (m, 6H, Ar) ppm. Anal. ($C_{21}H_{20}BrNO_4$) C, H, N.

2,2-dimethyl-4'-(phenylsulfonyl)-2,3-dihydro-5'H-spiro[chromene-4,2'-[1,4]oxazinan]-5'-one 25a.
Compound **25a** was synthesized from **52a** (0.2 g, 0.8 mmol) and benzenesulfonyl chloride (0.14 g, 0.8 mmol) following the same procedure described above for **24a.** The crude product was purified by trituration with Hexane to give **25a** (0.03 g, 0.08 mmol, yield 10%). mp162-167 ˚C. ^{1}H-NMR ($CDCl_3$) δ: 1.36 (s, 3H, Me); 1.44 (s, 3H, Me); 2.04 (d, 1H, *J* = 14.6 Hz, CH_2); 2.24 (d, 1H, *J* = 14.6 Hz, CH_2); 4.08 (d, 1H, *J* = 12.9 Hz, CH_2); 4.17 (d, 1H, *J* = 12.9 Hz, CH_2); 4.20 (d, 1H, *J* = 18 Hz, CH_2); 4.32 (d, 1H, *J* = 18 Hz, CH_2);

6.87-6.98 (m, 2H, Ar); 7.23-7.36 (m, 2H, Ar); 7.55-7.75 (m, 3H, Ar); 8.06–8.10 (m, 2H, Ar) ppm. ^{13}C NMR (CDCl3) *δ*: 166.80, 153.69, 137.99, 134.50, 130.75, 129.09, 128.91, 126.92, 121.00, 118.58, 74.36, 70.24, 64.47, 54.69, 40.01, 29.31, 25.94. Anal. ($C_{20}H_{21}NO_5S$) C, H, N.

6-bromo-2,2-dimethyl-4'-(phenylsulfonyl)-2,3-dihydro-5'H-spiro[chromene-4,2'-[1,4]oxazinan]-5'-one 25b.
Compound **25b** was synthesized from **49b** (0.2 g, 0.61 mmol) and benzenesulfonyl chloride (0.11 g, 0.61 mmol) following the same procedure described above for **24a**. The crude product was purified by flash column chromatography eluted by Hex/EtOAc (4/1) to give **25b** (0.11 g, 0.23 mmol, yield 38%). ^{1}H-NMR ($CDCl_3$) δ: 1.35 (s, 3H, Me); 1.44 (s, 3H, Me); 2.05 (d, 1H, *J* = 14.6 Hz, CH_2); 2.24 (d, 1H, *J* = 14.6 Hz, CH_2); 3.99 (d, 1H, *J* = 13.0 Hz, CH_2); 4.16-4.37 (m, 3H, CH_2); 6.78 (d, 1H, *J* = 8.8 Hz, Ar); 7.33-7.38 (m, 1H, Ar); 7.47 (d, 1H, *J* = 2.38, Ar); 7.55-7.71 (m, 3H, Ar); 8.06–8.10 (m, 2H, Ar) ppm. ^{13}C NMR ($CDCl_3$) δ: 166.62, 152.99, 137.95, 134.74, 133.78, 129.92, 129.33, 129.24, 129.04, 120.59, 113.13, 75.07, 70.27, 64.60, 54.57, 39.75, 29.24, 26.00. Anal. ($C_{20}H_{20}BrNO_5S$) C, H, N.

(2,2-dimethyl-2,3-dihydro-4'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl) (phenyl)methanone 26a.
To a solution of **53a** (0.3 g, 1.29 mmol) in CH_2Cl_2 was added triethylamine (0.15 g, 1.55 mmol) and benzoyl chloride (0.18 g, 1.29 mmol). The resulting mixture was stirred rt overnight and then CH_2Cl_2 was added and the organic phase was washed with HCl 1N, $NaHCO_3$, brine. The organic layer was dried, filtered and evaporated under vacuum. The crude product was purified by precipitation from iPr_2O/Hex to give **26a** (0.21 g, 0.63 mmol, yield 49%): mp110-116°C. ^{1}H-NMR $(CD_3)_2CO$ δ: 1.33 (br s, 7H, CH_2, Me); 2.47 (d, 1H, *J* = 14.5 Hz, CH_2); 3.21–3.88 (m, 5H, CH_2); 3.97 (dt, 1H, *J* = 3, 11.9 Hz, CH_2); 6.58–6.83 (m, 1H, Ar); 6.90-6.97 (m, 1H, Ar); 7.16-7.23 (m, 1H, Ar); 7.32–7.72 (m, 6H, Ar) ppm. ^{13}C NMR $(CD_3)_2CO$ δ: 168.94, 153.00, 135.38, 128.93, 128.81, 127.75, 127.40, 126.60, 123.18, 119.56, 116.86, 73.73, 69.27, 59.11, 41.48, 27.04, 25.42. Anal. ($C_{21}H_{23}NO_3$) C, H, N.

(6-bromo-2,2-dimethyl-2,3-dihydro-4'H-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl)(phenyl)methanone 26b.
Compound **26b** was synthesized from **53b** (0.37 g, 1.18 mmol) and benzoyl chloride (0.15g, 1.07 mmol) following the same procedure described above for **26a**. The crude product was purified by flash column chromatography eluted with Hex/Ac 4/1 to give **26b** (0.12 g, 0.29 mmol, yield 25%) mp 99-101 ºC. ^{1}H-NMR (CD_3OD) δ: 1.27–1.47 (m, 6H, Me); 2.04–2.45 (m, 2H, CH_2); 3.48–3.95 (m, 5H, CH_2); 4.47–4.53 (m, 1H, CH_2); 6.71–6.78 (m, 1H, Ar) 7.34–7.73 (m, 7H, Ar) ppm.

^{13}C NMR $(CD_3)_2CO$ δ: 168.94, 152.35, 135.25, 131.68, 130.15, 128.99, 127.77, 126.62, 119.14, 111.11, 74.45, 69.25, 59.25, 37.46, 26.79, 25.53. Anal. ($C_{21}H_{22}BrNO_3$) C, H, N.

(2,2-dimethyl-2,3-dihydro-4'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl)(4-methylphenyl)methanone 27a.
Compound **27a** was synthesized from **53a** (0.2 g, 0.858 mmol) and p-toluoyl chloride (0.12g, 0.77 mmol) following the same procedure described above for **26a**. The crude product was purified by flash column chromatography eluted with Hex/EtOAc (4/1) to give **27a** (0.1 g, 0.28 mmol, yield 33%). ^{1}H-NMR $(CD_3)_2CO$ δ: 1.33 (br s, 7H, CH_2, Me); 2.34 (s, 3H, Me); 2.46 (d, 1H, *J* = 14.8 Hz, CH_2); 3.21–3.88 (m, 5H, CH_2); 3.97 (dt, 1H, *J* = 12, 2.9 Hz, CH_2); 6.75 (d, 1H, *J* = 8.42 Hz, Ar); 6.89-6.97 (m, 1H, Ar); 7.16-7.20 (m, 1H, Ar); 7.25 (d, 2H, *J* = 7.87 Hz, Ar); 7.37 (d, 2H, *J* = 7.87 Hz, Ar); 7.59 (d, 1H, *J* = 7.14 Hz, Ar) ppm. ^{13}C NMR ($CDCl_3$) δ: 160.25, 153.75, 140.55, 132.32, 130.15, 129.48, 127.51, 120.94, 118.26, 74.67, 70.36, 64.99, 59.87, 57.83, 38.75, 28.55, 26.06, 21.74. Anal. ($C_{22}H_{25}NO_3$) C, H, N.

(2,2-dimethyl-2,3-dihydro-4'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl)(4-fluorophenyl)methanone 28a.
To a solution of **53a** (0.2 g; 0.86 mmol) in THF was added 4-fluorobenzoic acid (0.12 g; 0.86 mmol); *N*,*N*'-dicyclohexylcarbodiimide (0.21 g; 1.03 mmol) and catalytic amount of 4-(dimethylamino)pyridine. The reaction mixture was stirred rt for 1h and then the solvent was evaporated under vacuum. The crude product was purified by flash column chromatography eluted by Hex/EtOAc (1/1) to give **28a** (0.19g, 0.55 mmol, yield 64%). ^{1}H-NMR $(CD_3)_2CO$ δ: 1.33 (br s, 7H, CH_2, Me); 2.46 (d, 1H, *J* = 14.6 Hz, CH_2); 3.30–3.80 (m, 5H, CH_2); 3.97 (dt, 1H, *J* = 2.9, 11.9 Hz, CH_2); 6.76 (d, 1H, *J* = 8.06 Hz, Ar); 6.89-6.97 (m, 1H, Ar); 7.16-7.25 (m, 3H, Ar); 7.53-7.60 (m, 3H, Ar); ppm. ^{13}C-NMR ($CDCl_3$) δ: 170.06, 153.74, 130.04, 129.74, 129.58, 127.40, 123.23, 120.79, 118.24, 116.11, 115.67, 74.54, 70.37, 60.03, 40.47, 40.40, 39.03, 28.47, 25.10. Anal. ($C_{21}H_{22}FNO_3$) C, H, N.

(2,2-dimethyl-2,3-dihydro-4'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl) (phenyl)methanethione 29a.
Compound **29a** was synthesized from **26a** (0.21 g, 0.62 mmol) and Lawesson's Reagent (0.15 g, 0.37 mmol) following the same procedure described above for **21a**. The crude product was purified by flash column chromatography eluted by Hex/EtOAc (4/1) to give **32a** (0.05 g, 0.15 mmol, yield 24%). mp 132-137 ºC. ^{1}H-NMR ($CDCl_3$) δ: 1.19 (s, 3H, CH_3); 1.30 (s, 3H, CH_3); 1.58-1.61 (m, 1H, CH_2); 2.36 (d, 1H, *J* = 19.9 Hz, CH_2); 3.43-4.12 (m, 6H, CH_2); 6.77 (d, 1H, *J* = 7.9 Hz, Ar); 6.86-6.94 (m, 1H, Ar); 6.99-7.03 (m, 1H, Ar); 7.17-7.55 (m, 6H, Ar) ppm. Anal. ($C_{21}H_{23}NO_2S$) C, H, N.

(4-aminophenyl)(2,2-dimethyl-2,3-dihydro-4'*H*-spiro[chromene-4,2'-[1,4] oxazinan]-4'-yl)methanone 30a.
Compound **30a** was synthesised from **51a** (0.33 g; 1.43 mmol) and p-aminobenzoic acid (0.19 g; 1.43 mmol) and *N*,*N*'-dicyclohexylcarbodiimide (0.35 g; 1.7 mmol) and catalytic amount of 4-(dimethylamino)pyridine. The reaction mixture was stirred overnight and then the solvent was evaporated under vacuum. The crude product was purified by flash column chromatography eluted by Hex/EtOAc (1/1) to give **30a** (0.17 g, 0.47 mmol, yield 33%). ^{1}H-NMR $(CD_3)_2CO$ δ: 1.32 (br s, 6H, Me); 1.95 (d, 1H, *J* = 14.6 Hz, CH_2); 2.39 (d, 1H, *J* = 14.6 Hz, CH_2); 3.30–3.44 (m, 1H, CH_2); 3.56 (d, 1H, *J* = 13.0 Hz, CH2); 3.69 (dd, 1H, *J* = 2.6, 11.9 Hz, CH_2); 3.87-4.15 (m, 3H, CH2) 5.05 (br s, 2H, CH_2); 6.66 (d, 2H, *J* = 8.5 Hz, Ar); 6.72–6.77 (m, 1H, Ar); 6.89-6.97 (m, 1H, Ar); 7.15-7.19 (m, 1H, Ar); 7.24 (d, 2H, *J* = 8.5 Hz, Ar); 7.59 (dd, 1H, *J* = 1.6, 7.8 Hz, Ar) ppm. ^{13}C NMR $(CD_3)_2CO$ δ: 169.76, 153.00, 149.76, 128.79, 128.71, 127.44, 123.42, 122.23, 119.54, 116.82, 112.51, 73.74, 69.32, 59.09, 37.86, 26.95, 25.38. Anal. ($C_{21}H_{24}N_2O_3$) C, H, N.

***N*-{4-[(2,2-dimethyl-2,3-dihydro-4'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl)carbonyl]phenyl}acetamide 31a.**
To a solution of **30a** (0.22 g; 0.61 mmol) in acetone (5mL) was added K_2CO_3 (0.13 g, 0.91 mmol) and acetic anhydride (0.06 g, 0.61 mmol) and then the reaction mixture was stirred rt overnight. The solvent was evaporated and the residue was dissolved in EtOAc, washed with water and brine. The organic layer was dried and concentrated under vacuum. Th crude product was purified by flash column chromatography eluted by Hex/EtOAc (3/7) to give **31a** (0.12g, 0.3 mmol, yield 50%). ^{1}H-NMR ($CDCl_3$) δ : 1.34 1.32 (br s, 7H, CH_2, Me); 2.18 (s, 3H, Me); 2.33 (d, 1H, *J* = 14.5 Hz, CH_2); 3.37 (br s, 2H, CH_2); 3.79 (br s, 4H, CH_2); 6.80 (d, 1H, *J* = 8.5 Hz, Ar); 6.92–6.99 (m, 1H, Ar); 7.17-7.21 (m, 1H, Ar); 7.36-755 (m, 5H, Ar) ppm. ^{13}C NMR $(CD_3)_2CO$ δ: 168.86, 167.46, 153.06, 140.31, 129.70, 128.68, 127.55, 127.29, 123.38, 119.50, 117.86, 116.86, 73.71, 69.36, 59.07, 38.11, 25.29, 22.80. Anal. ($C_{23}H_{26}N_2O_4$) C, H, N.

***N*-{4-[(2,2-dimethyl-2,3-dihydro-4'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl)carbonyl]phenyl}methanesulfonamide 32a.**
To a solution of **30a** (0.22 g; 0.61 mmol) in dioxane (5mL) was added K_2CO_3 (0.13 g, 0.91 mmol) and acetic anhydride (0.06 g, 0.61 mmol) and then the reaction mixture was stirred rt overnight. The solvent was evaporated and the residue was dissolved in EtOAc, washed with water and brine. The organic layer was dried and concentrated under vacuum. The crude product was purified by flash column chromatography eluted by Hex/EtOAc (3/7) to give **32a** (0.12g, 0.3 mmol, yield 50%).^{1}H-NMR $(CD_3)_2CO$ δ: 1.33 (br s, 7H, CH_2,

Me); 2.44 (d, 1H, *J* = 14.6 Hz, CH_2); 3.02 (s, 3H, Me); 3.21–3.74(m, 5H, CH_2); 3.97 (dt, 1H, *J* = 2.8, 11.9 Hz, CH_2); 6.76 (d, 1H, *J* = 8.06 Hz, Ar); 6.89-6.97 (m, 1H, Ar); 7.15-7.24 (m, 1H, Ar); 7.38 (d, 2H, *J* = 8.5 Hz, Ar); 7.50 (d, 2H, *J* = 8.5 Hz, Ar); 7.59 (dd, 1H, *J* = 7.7, 1.5 Hz, Ar) ppm. ^{13}C-NMR ($CDCl_3$) δ: 170.36, 153.82, 139.31, 131.28, 130.15, 129.13, 127.51, 120.90, 119.72, 118.39, 118.30, 74.67, 70.47, 65.66, 60.05, 39.97, 39.60, 39.08, 34.09, 28.64. Anal. ($C_{22}H_{26}NO_2S$) C, H, N.

2,2-dimethyl-4'-(phenylsulfonyl)-2,3-dihydro-5'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-5'-one 33a.

Compound **33a** was obtained from **51a** (0.3 g, 1.28mmol) and benzenesulfonyl chloride (0.22 g, 1.28 mmol) following the same procedure described above for **26a**. The crude product was purified by crystallization from *i*Pr_2O to give **33a** (0.05 g, 0.12 mmol, yield 10%) mp135-140 C. ^{1}H-NMR ($CDCl_3$) δ: 1.39 (s, 3H, Me); 1.44 (s, 3H, Me); 2.32 (d, 1H, *J* = 14.7 Hz, CH_2); 2.41 (d, 1H, *J* = 14.7 Hz, CH_2); 2.55–2.69 (m, 2H, CH_2); 3.54–3.67 (m, 2H, CH_2); 3.76–3.83 (m, 1H, CH_2); 4.02 (dt, 1H, *J* = 2.8, 11.9 Hz, CH_2); 6.82-6.92 (m, 2H, Ar); 7.17–7.33 (m, 2H, Ar); 7.52–7.75 (m, 5H, Ar) ppm. ^{13}C-NMR ($CDCl_3$) δ: 153.95, 136.16, 133.21, 130.06, 129.41, 127.86, 127.44, 123.34, 120.69, 118.32, 74.95, 70.16, 60.22, 55.59, 46.09, 39.51, 28.50, 27.70. Anal. ($C_{20}H_{23}NO_4S$) C, H, N.

6-bromo-2,2-dimethyl-4'-(phenylsulfonyl)-2,3-dihydrospiro[chromene-4,2'-[1,4]oxazinane] 33b

Compound **33b** was obtained from **51b** (0.15g, 0.48 mmol) and benzenesulfonyl chloride (0.08 g, 0.48 mmol) following the same procedure described above for **26a**. The crude product was purified by flash column chromatography eluted by Hex/EtOAc (4/1) to give **33b** (0.03g, 0.06 mmol, yield 14%).^{1}H-NMR ($CDCl_3$) δ : 1.38 (s, 3H, Me); 1.43 (s, 3H, Me); 2.28 (d, 1H, *J* = 14.5 Hz, CH_2); 2.44 (d, 1H, *J* = 14.5 Hz, CH_2); 2.53–2.67 (m, 2H, CH_2); 3.58–3.83 (m, 3H, CH_2); 3.96–4.07 (m, 1H, CH_2); 6.72 (d, 1H, *J* = 8.6 Hz, Ar); 7.26–7.31 (m, 1H, Ar); 7.39 (d, 1H, *J* = 2.5 Hz, Ar); 7.55–7.75 (m, 5H, Ar) ppm. ^{13}C-NMR ($CDCl_3$) δ : 153.04, 135.69, 133.56, 133.08, 130.28, 129.66, 127.82, 125.33, 120.25, 112.80, 75.60, 70.07, 60.34, 55.46, 46.01, 38.91, 28.17, 27.86. Anal. ($C_{20}H_{22}BrNO_4S$) C, H, N.

(2,2-dimethyl-5'-oxo-2,3-dihydro-4'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl)acetic acid 34a.

To a stirred solution of **55a** (0.26 g, 0.77 mmol) in MeOH (3 mL) was added KOH 50% (0.005 mL) and the resulting mixture was refluxed for 2h. The solvent was evaporated under vacuum and then HCl 1N was added (pH =3). The precipitate was filtered and dried to give **34a** (0.14 g, .0.46 mmol, 60% yield). mp 163-165˚C. ^{1}H-NMR ($CDCl_3$) δ : 1.41 (s, 6H, Me); 2.19 (d, 1H, *J* = 14.6 Hz, CH_2); 2.42 (d, 1H, *J* = 14.6 Hz, CH_2); 3.26 (d,1H, *J* = 12.08 Hz, CH_2);

4.02- 4.10 (m, 2H, CH_2); 4.35- 4.46 (m, 3H, CH_2); 6.84- 7.0 (m, 2H, Ar); 7.22- 7.29 (m, 1H, Ar); 7.46- 7.47 (m, 1H, Ar) ppm. Anal. ($C_{16}H_{19}NO_5$) C, H, N.

(6-bromo-2,2-dimethyl-5'-oxo-2,3-dihydro-4'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl)acetic acid 34b.
Compound **34b** was obtained from **55b** (0.39 g, 0.96 mmol) following the same procedure descrided for **34a**. The crude product was collected without further purification to yield **34b** (0.12 g, 0.32 mmol, 34%yield). mp 132-134˚C. ^{1}H-NMR (DMSO) δ : 1.32 (s, 3H, Me); 1.35 (s, 3H, Me); 2.14 (d, 1H, *J* = 15 Hz, CH_2); 2.42- 2.52 (m, 1H, CH_2); 3.96- 4.24 (m, 6H, CH_2); 6.79 (d, 1H, *J* = 8.6 Hz, Ar); 7.4 (dd, 1H, *J* = 2.5, 8.7 Hz, Ar); 7.6 (d, 1H, *J* = 2.4 Hz, Ar) ppm. Anal. ($C_{16}H_{18}BrNO_5$) C, H, N.

(2,2-dimethyl-2,3-dihydro-4'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl)acetic acid 35a.
Compound **35a** was obtained from **56a** (0.55 g, 1.90 mmol) following the same procedure descrided for **34a**. The crude product was collected and purified by precipitation from $CHCl_3$/Hex to yield **35a** (0.01 g, 0.04 mmol, yield 2%). ^{1}H-NMR (CD_3OD) δ : 1.35 (s, 3H, Me); 1.45 (s, 3H, Me); 2.20 (d, 1H, *J* = 14.8 Hz, CH_2); 2.65 (d, 1H, *J* = 14.8 Hz, CH_2); 3.26-3.40 (m, 2H, CH_2); 3.70-4.01 (m, 3H, CH_2); 4.07-4.24 (m, 1H, CH_2); 4.90 (s, 2H, CH_2); 6.78- 6.83 (m, 1H, Ar); 6.93- 7.00 (m, 1H, Ar); 7.20- 7.28 (m, 1H, Ar); 7.55 (dd, 1H, *J* = 1.5, 7.9 Hz, Ar) ppm. Anal. ($C_{16}H_{21}NO_4$) C, H, N.

(6-bromo-2,2-dimethyl-2,3-dihydro-4'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl)acetic acid 35b.
Compound **35b** was obtained from **53b** (0.03 g, 0.11 mmol) and chloroacetic acid (0.01 g, 0.11 mmol) following the same procedure described above for **18a**. compound **35b** was obtained without further purification. **35b** (0.005 g, 0.07 mmol, yield 67%). ^{1}H-NMR ($CDCl_3$) δ : 1.33 (s, 3H, Me); 1.40 (s, 3H, Me); 2.14 (d, 1H, *J* = 14.5 Hz, CH_2); 2.54 (d, 1H, *J* = 14.5 Hz, CH_2); 2.70-3.00 (m, 4H, CH_2); 3.19-3.42 (m, 2H, CH_2); 3.78-3.84 (m, 1H, CH_2); 3.99-4.10 (m, 1H, CH_2); 6.71 (d, 1H, *J* = 8.8 Hz, Ar); 7.29 (dd, 1H, *J* = 2.4, 8.8 Hz, Ar); 7.64 (d, 1H, *J* = 2.4 Hz, Ar) ppm. Anal. ($C_{16}H_{20}BrNO_4$) C, H, N.

(2,2-dimethyl-2'-oxo-2,3-dihydro-3'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl)acetic acid 36a.
Compound **36a** was obtained from **55a** (0.23 g, 0.56 mmol) following the same procedure descrided for **34a** without further purification. **36a** (0.04 g, 0.16 mmol, yield 29%). mp 160-165˚C. ^{1}H-NMR (DMSO) δ : 1.32 (s, 3H, Me); 1.36 (s, 3H, Me); 2.27 (d, 1H, *J* = 14.8 Hz, CH_2); 2.37 (d, 1H, *J* = 14.8 Hz, CH_2); 3.68 (d, 1H, *J* = 9.1 Hz, CH_2); 3.87 (d, 1H, *J* = 9.1 Hz, CH_2); 3.95 (d, 1H, *J* = 17.7 Hz, CH_2); 4.08 (d, 1H, *J* = 17.7 Hz, CH_2); 6.8 (d, 1H, *J* = 8.7 Hz, Ar); 6.98

(m, 1H, Ar); 7.26 (m, 1H, Ar); 7.58 (d, 1H, *J* = 7.7 Hz, Ar) ppm. Anal. ($C_{15}H_{17}NO_5$) C, H, N.

(6-bromo-2,2-dimethyl-2'-oxo-2,3-dihydro-3'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl)acetic acid 36b.
Compound **36b** was obtained from **55b** (0.33 g, 0.67 mmol) following the same procedure descrided for **34a** without further purification**. 36b** (0.12 g, 0.35 mmol, 53%yield). mp165-170°C ^{1}H-NMR (DMSO) δ : 1.32 (s, 3H, Me); 1.36 (s , 3H, Me) ; 2.27 (d , 1H , *J* = 15.0 Hz, CH_2) ; 2.37 (d, 1H , *J* = 15.0 Hz, CH_2); 3.68 (d, 1H, *J* = 9.2 Hz, CH_2); 3.88 (d, 1H, *J* = 9.2 Hz, CH_2); 3.90 (d, 1H, *J* = 18.1 Hz, CH_2); 4.0 (d, 1H, *J* = 18.1 Hz, CH_2); 6.79 (d, 1H, *J* = 8.7 Hz, Ar); 7.43 (dd, 1H, *J* = 2.3, 8.7 Hz, Ar); 7.85 (d, 1H, *J* = 2.3 Hz, Ar) ppm. Anal. ($C_{15}H_{16}BrNO_5$) C, H, N.

[2-(4-methoxyphenyl)-2-methyl-2'-oxo-2,3-dihydro-3'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetic acid 37a.
Compound **37a** was obtained from **64a** (0.03 g, 0.07 mmol) following the same procedure descrided for **34a** without further purification. **37a** (0.006 g, 0.01 mmol, yield 22%). ^{1}H-NMR (CD_3OD) δ : 1.68 (s, 3H, Me); 2.25 (d, 1H, *J* = 12.6 Hz, CH_2); 2.41 (d, 1H, *J* = 14.3 Hz, CH_2); 2.83 (d, 1H, *J* = 14.3 Hz, CH_2); 2.96 (d, 1H, *J* = 12.6 Hz, CH_2); 3.11-3.31 (m, 2H, CH_2); 3.76 (s, 3H, OMe); 6.817- 7.04 (m, 4H, Ar);7.263- 7.40 (m, 3H, Ar); 7,46- 7.51 (m, 1H, Ar) ppm. Anal. ($C_{21}H_{21}NO_6$) C, H, N.

[6-bromo-2-(4-methoxyphenyl)-2-methyl-2'-oxo-2,3-dihydro-3'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetic acid 37b.
Compound **37b** was obtained from **64b** (0.25 g, 0.51 mmol) following the same procedure descrided for **34a** without further purification. **37b** (0.1 g, 0.23 mmol, 45%yield). mp78-80°C ^{1}H-NMR (DMSO) δ :1.63 (s, 3H, Me); 2.69 (d, 2H, *J* = 17 Hz, CH_2); 3.30-3.40 (m, 2H); 3.73 (s, 3H, OMe); 3.86 (d, 1H, *J* = 17.9 Hz, CH_2); 4.00 (d, 1H, *J* = 17.9 Hz, CH_2); 6.90 (d, 2H, *J* = 8.7 Hz, Ar); 6.99- 7.06(m, 1H, Ar); 7.26- 7.38 (m, 2H, Ar); 7.5 (dd, 1H, *J* = 2.4; 8.7 Hz, Ar); 7.76 (d, 1H, *J* = 2.4 Hz, Ar) ppm. Anal. ($C_{21}H_{20}BrNO_6$) C, H, N.

[2-(4-chlorophenyl)-2-methyl-2'-oxo-2,3-dihydro-3'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetic acid 38a.
Compound **38a** was obtained from **65a** (0.39 g, 0.95 mmol) following the same procedure descrided for **34a**. The crude product was collected and purified by precipitation from EtOAC/Hex to yield **38a** (0.2 g, 0.52 mmol, yield 55%): mp 85-87°C. ^{1}H-NMR ($CDCl_3$) δ : 1.72 (s, 3H, Me); 2.58 (d, 1H, *J* = 14.5 Hz, CH_2); 2.72 (d, 1H, *J* = 14.5 Hz, CH_2); 3.26 (d, 1H, *J* = 8.8 Hz, CH_2); 3.50 (d, 1H, *J* = 8.8 Hz, CH_2); 4.05 (s, 2H, CH_2); 6.96-7.05 (m, 2H, Ar); 7.27-7.51 (m, 6H, Ar) ppm. Anal. ($C_{20}H_{18}ClNO_5$) C, H, N.

[6-bromo-2-(4-chlorophenyl)-2-methyl-2'-oxo-2,3-dihydro-3'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetic acid 38b.
Compound **38b** was obtained from 65b (0.47 g, 0.95 mmol) following the same procedure descrided for **34a**. The crude product was collected and purified by precipitation from EtOAC/Hex to yield **38b** (0.27 g, 0.59 mmol, yield 62%): mp103-106°C. ^{1}H-NMR ($CDCl_3$) δ : 1.71 (s, 3H, Me); 2.56 (d, 1H, *J* = 14.5 Hz, CH_2); 2.70 (d, 1H, *J* = 14.5 Hz, CH_2); 3.18-3.52 (m, 2H, CH_2); 3.95-4.17 (m, 2H, CH_2); 6.89-7.04 (m, 1H, Ar); 7.26-7.52 (m, 5H, Ar); 7.69 (d, 1H, *J* = 2.0 Hz, Ar) ppm. Anal. ($C_{20}H_{17}BrClNO_5$) C, H, N.

2-(4-methoxyphenyl)-2-methyl-5'-oxo-2,3-dihydro-4'H-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl)acetic acid 39a.
Compound **39a** was obtained from **68a** (0.47 g, 1.10 mmol**)** following the same procedure descrided for **34a** without further purification. **39a** (0.09 g, 0.23 mmol, yield 21%). mp175-178°C ^{1}H-NMR (DMSO) δ : 1.63 (s, 1H, Me); 2.30 (d, 1H, *J* = 12.6 Hz, CH_2); 2.42 (d, 1H, *J* = 14.3 Hz, CH_2); 2.92 (d, 1H, *J* = 14.3 Hz, CH_2); 3.25 (d, 1H, *J* = 17.2 Hz, CH_2); 3.62 (d, 1H, *J* = 12.6 Hz, CH_2); 3.71 (s, 3H, OMe); 4.16 (d, 1H, *J* = 17.3 Hz, CH_2); 4.22 (d, 1H, *J* = 17.2 Hz, CH_2); 4.35 (d, 1H, *J* = 17.2 Hz, CH_2); 6.88 (d, 2H, *J* = 8.7 Hz, Ar); 6.94-7.04 (m, 2H, Ar); 7.26-7.42 (m, 4H, Ar) ppm. Anal. ($C_{22}H_{23}NO_6$) C, H, N.

[6-bromo-2-(4-methoxyphenyl)-2-methyl-5'-oxo-2,3-dihydro-4'H-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl)acetic acid 39b.
Compound **39b** was obtained from **68b** (0.2 g, 0.4 mmol) following the same procedure descrided above for **34a**. The crude product was purified by crystallization from (iPr)$_2$O to give **39b** (0.03 g, 0.07 mmol, yield 18%) ^{1}H-NMR (DMSO) δ : 1.64 (s, 1H, Me); 2.30-2.39 (m, 1H, CH_2); 2.92 (d, 1H, *J* = 14.6 Hz, CH_2); 2.92 (d, 1H, *J* = 14.3 Hz, CH_2); 3.25-3.38 (m, 2H, CH_2); 3.54-3.65 (m, 1H, CH_2); 3.72 (s, 3H, OMe); 4.08-4.40 (m, 3H, CH_2); 6.89 (d, 2H, *J* = 8.8 Hz, Ar); 7.03 (d, 1H, *J* = 8.6 Hz, Ar); 7.29-7.35 (m, 2H, Ar); 7.45-7.50 (m, 1H, Ar); 7.55 (d, 1H, *J* = 2.4 Hz, Ar) ppm. Anal. ($C_{22}H_{22}BrNO_6$) C, H, N.
[2-(4-methoxyphenyl)-2'-oxo-2,3-dihydro-3'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetic acid 40a.
Compound **40a** was obtained from **91a** (0.03g, 0.07 mmol) following the same procedure descrided above for **34a**. **40a** (0.02 g, 0.05 mmol, yield 72%) ^{1}H-NMR ($CDCl_3$) δ : 2.45-2.65 (m, 2H, CH_2); 3.70-3.93 (m, 5H, CH_2, OMe); 4.12 (s, 2H, CH_2); 4.98-5.04 (m, 1H, CH); 6.88-7.03 (m, 4H, Ar); 7.22--7.27 (m, 1H, Ar); 7.37 (d, 2H, *J* = 8.0 Hz, Ar); 7.46-7.49 (m, 1H, Ar) ppm. Anal. ($C_{20}H_{19}NO_6$) C, H, N.

[6-bromo-2-(4-methoxyphenyl)-2'-oxo-2,3-dihydro-3'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetic acid 40b.
Compound **40b** was obtained from **91b** (0.17 g, 0.37 mmol) following the same procedure descrided for **34a**. **40b** (0.1 g, 0.22 mmol, 60 %yield). ^{1}H-NMR (CD_3OD) δ : 2.30-2.60 (m, 2H, CH_2); 3.81 (s, 3H, OMe); 3.90 (d, 1H, *J* = 9.5 Hz, CH_2); 3.99 (d, 1H, *J* = 9.5 Hz, CH_2); 4.04 (d, 1H, *J* = 18 Hz, CH_2); 4.14 (d, 1H, *J* = 18 Hz, CH_2); 5.15 (dd, 1H, *J* = 2.2, 11.7 Hz, CH); 6.82 (d, 1H, *J* = 8.8 Hz, Ar); 6.96 (d, 2H, *J* = 8.8 Hz, Ar); 7.36-7.44 (m, 3H, Ar); 7.63 (d, 1H, *J* = 2.4 Hz, Ar) ppm. Anal. ($C_{20}H_{18}BrNO_6$) C, H, N.

[2-(4-chlorophenyl)-2'-oxo-2,3-dihydro-3'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetic acid 41a.
Compound **41a** was obtained from **92a** (0.05 g, 0.11 mmol) following the same procedure descrided for **34a**. **41a** (0.04 g, 0.10 mmol, 99 %yield). ^{1}H-NMR (CD_3OD) δ : 2.40-2.65 (m, 2H, CH_2); 3.90-4.23 (m, 4H, CH_2); 4.88 (s, 2H, CH_2); 5.19-5.25 (m, 1H, CH); 6.89-7.08 (m, 2H, Ar); 7.24-7.33 (m, 1H, Ar); 7.40-7.54 (m, 5H, Ar) ppm. Anal. ($C_{19}H_{16}ClNO_5$) C, H, N.

[6-bromo-2-(4-chlorophenyl)-2'-oxo-2,3-dihydro-3'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetic acid 41b
Compound **41b** was obtained from **92b** (0.32 g, 0.68 mmol) following the same procedure described for **34a**.The crude product was purified by trituration with Hexane to give **41b** (0.1 g, 0.23 mmol, yield 34%): mp153-155 °C. ^{1}H-NMR ($CDCl_3$) δ : 2.45-2.52 (m, 2H, CH_2); 3.79-3.92 (m, 2H, CH_2); 4.11 (s, 2H, CH_2); 4.98-5.08 (m, 1H, CH); 6.79 (d, 1H, *J* = 9.0 Hz, Ar); 6.89-7.05 (m, 1H, Ar); 7.30-7.49 (m, 4H, Ar); 7.59 (d, 1H, *J* = 2.2 Hz, Ar) ppm. Anal. ($C_{19}H_{15}BrClNO_5$) C, H, N.

[2-(2,4-dichlorophenyl)-2'-oxo-2,3-dihydro-3'H-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetic acid 42a
Compound **42a** was obtained from **93a** (0.05 g, 0.11 mmol) following the same procedure descrided for **34a**. The crude product was collected and purified by crystallization from iPrOH to yield **42a** (0.008 g, 0.02 mmol, yield 19%). mp128-130 ˚C. ^{1}H-NMR ($CDCl_3$) δ : 2.17-2.37 (m, 1H, CH_2); 2.62-2.71 (m, 1H, CH_2); 3.55-4.17 (m, 4H, CH_2); 5.38-5.50 (m, 1H, CH); 6.93-7.10 (m, 1H, Ar); 7.24-7.74 (m, 5H, Ar) ppm. Anal. ($C_{19}H_{15}Cl_2NO_5$) C, H, N.

[6-bromo-2-(2,4-dichlorophenyl)-2'-oxo-2,3-dihydro-3'H-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetic acid 42b
Compound **42b** was obtained from **93b** (0.38 g, 0.73 mmol) following the same procedure descrided for **34a**. The crude product was collected and purified by precipitation from EtOAC/Hex to yield **42b** (0.16 g, 0.33 mmol, yield 36%). mp190-192 ˚C. ^{1}H-NMR (DMSO) δ : 2.28- 2.41 (m, 1H, CH_2); 2.58 (d, 1H, *J* =

13.2 Hz, CH_2); 3.75-3.97 (m, 2H, CH_2); 4.01 (d, 2H, *J* = 2.8 Hz, CH_2); 5.58 (d, 1H, *J* = 11.5 Hz, CH); 6.96 (dd, 1H, *J* = 1.1, 8.8 Hz, Ar); 7.47-7.77 (m, 5H, Ar) ppm. Anal. ($C_{19}H_{14}BrCl_2NO_5$) C, H, N.

[6-bromo-2-(2,4-difluorophenyl)-2'-oxo-2,3-dihydro-3'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetic acid 43b.
Compound **43b** was obtained from **94b** (0.09 g, 0.18 mmol) following the same procedure descrided for **34a.** The crude product was collected and purified by trituration with Hexane to yield **43b** (0.05 g, 0.11 mmol, 63% yield): mp118-120 ˚C. ^{1}H-NMR ($CDCl_3$) δ : 2.39-2.58 (m, 2H, CH_2); 3.84 (d, 1H, *J* = 8.5 Hz, CH_2); 3.91 (d, 1H, *J* = 8.5 Hz, CH_2); 4.16 (s, 2H, CH_2); 5.31-5.37 (m, 1H, CH); 6.81 (d, 1H, *J* = 8.8 Hz, Ar); 6.86-7.01 (m, 2H, Ar); 7.37 (dd, 1H, *J* = 8.8, 2.4 Hz, Ar); 7.51-7.59 (m, 1H, Ar); 7.62 (d, 1H, *J* = 2.4 Hz, Ar) ppm. Anal. ($C_{19}H_{14}BrF_2NO_5$) C, H, N.

[6-bromo-2-(4-bromo-2-fluorophenyl)-2'-oxo-2,3-dihydro-3'*H*-spiro [chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetic acid 44b.
Compound **44b** was obtained from **95b** (0.38 g, 0.7 mmol) following the same procedure descrided for **34a** without further purification. **44b** (g, 0.21mmol, 30%yield). mp 178-180 ˚C. ^{1}H-NMR ($CDCl_3$) δ : 2.37-2.59 (m, 2H, CH_2); 3.81 (s, 2H, CH_2); 3.87 (d, 1H, *J* = 4.1 Hz; CH_2); 4.13 (d, 1H, *J* = 4.1 Hz, CH_2); 5.33 (d, 1H, *J* = 10.6 Hz, CH); 6.82 (d, 1H, *J* = 8.6 Hz, Ar); 7.26-7.53 (m, 4H, Ar); 7.61-7.62 (m, 1H, Ar) ppm. Anal. ($C_{19}H_{14}Br_2FNO_5$) C, H, N.

(5-{2-[(4-methoxybenzyl)oxy]phenyl}-5-methyl-2-oxo-1,3-oxazolidin-3-yl)acetic acid 45a
Compound **45a** was obtained from **114a** (0.05 g, 0.12 mmol) following the same procedure descrided for **34a.** The crude product was collected and purified by precipitation from EtOAc/Hex to yield **45a** (0.02 g, 0.06 mmol, 49% yield): mp117-119 ˚C. ^{1}H-NMR ($CDCl_3$) δ : 1.75 (s, 3H, Me); 3.67-3.79 (m, 2H, CH_2); 3.83 (s, 3H, OMe); 4.02-4.11 (m, 2H, CH_2); 5.00 (s, 2H, CH_2); 6.91-7.02 (m, 4H, Ar); 7.24-7.32 (m, 3H, Ar); 7.58 (dd, 1H, *J* = 1.8, 8.1 Hz, Ar) ppm. Anal. ($C_{20}H_{21}NO_6$) C, H, N.

(5-{2-[(3,4-dimethoxybenzyl)oxy]phenyl}-5-methyl-2-oxo-1,3-oxazolidin-3-yl)acetic acid 46a
Compound **46a** was obtained from **115a** (0.05 g, 0.12 mmol) following the same procedure descrided for **34a** without further purification. **46a** (0.04 g, 0.11 mmol, 90% yield): mp118-120 ˚C. ^{1}H-NMR ($CDCl_3$) δ : 1.79 (s, 3H, Me); 3.65-3.90 (m, 8H, CH_2, OMe); 4.03-4.17 (m, 2H, CH_2); 4.94-5.08 (m, 2H, CH_2); 6.83-7.04 (m, 5H, Ar); 7.26-7.33 (m, 1H, Ar); 7.59-7.63 (m, 1H, Ar) ppm. Anal. ($C_{21}H_{23}NO_7$) C, H, N.

(5-{2-[(3,4,5-trimethoxybenzyl)oxy]phenyl}-5-methyl-2-oxo-1,3-oxazolidin-3-yl)acetic acid 47a

Compound **47a** was obtained from **116a** (0.54g, 1.18 mmol) following the same procedure descrided for **34a**. The crude product was dissolved in EtOAc and washed with NaHCO3 and then exctracted with CH2Cl2. The organic layers were dried and concentrated under vacuum to give **47a** (0.07 g, 0.15 mmol, 13 % yield): ^{1}H-NMR ($CDCl_3$) δ : 1.83 (s, 3H, Me); 3.57-4.18 (m, 11H, CH_2, OMe); 5.02(s, 2H, CH_2); 6.61 (s, 2H, Ar); 6.95-7.05 (m, 2H, Ar); 7.26-7.34 (m, 1H, Ar); 7.59-7.63 (m, 1H, Ar) ppm. Anal. ($C_{22}H_{25}NO_8$) C, H, N.

2,2-dimethyl-2,3-dihydro-4*H*-chromen-4-one 48a.

To a solution of 2-hydroxy acetophenone (3.00 g, 22.00 mmol) in CH_3CN (20 mL) was added acetone (7.66 g, 132.00 mmoli) and pyrrolidine (3.72 g, 52.35 mmol). The mixture was stirred at rt for 1h, then refluxed for 8 h. The solvent was removed under reduced pressure and the residue diluted with EtOAc and washed with HCl 1N, NaOH 1N and water. The organic layers were dried and evaporated The crude product was directly used in the next step without further purification. **48a** (2.51 g, 14.3 mmol, yield 65%): ^{1}H-NMR ($CDCl_3$) δ : 1.46 (s, 6H, Me); 2.72 (s, 2H, CH_2); 6.95 (m, 2H, Ar); 7.46 (m, 1H, Ar); 7.85 (m, 1H, Ar). MS (*m/z*): 177 (M^+, 100%).

6-bromo-2,2-dimethyl-2,3-dihydro-4*H*-chromen-4-one 48b.

Compound **48b** was obtained from 5-bromo-2-hydroxy acetophenone (3.00 g, 13.95 mmol) following the procedure described for **48a**. The crude product was directly used in the next step without further purification. **48b** (2.88 g, 11.3 mmol, yield 81%): ^{1}H-NMR ($CDCl_3$) δ : 1.45 (s, 6H, Me); 2.71 (s, 2H, CH_2); 6.82 (d,1H, *J* = 8.8 Hz, Ar); 7.53 (dd, 1H, *J* = 8.8; 2.5 Hz, Ar); 7.95 (d, 1H, *J* = 2.5 Hz, Ar) ppm.

3,4-dihydro-2,2-dimethyl-4-[(trimethylsilyl)oxy]-2H-1-benzopyran-4-carbonitrile 49a.

To a solution of **48a** (1.23 g, 7 mmol) in CH_2Cl_2 was added trimethylsilylcyanide (TMSCN) (1.4 mL, 10.5 mmol) and ZnI_2 (0.33 g, 1.05 mmol). The mixture was stirred at r.t. for 4 h, then CH_2Cl_2 was added and the solution was washed with water. The organic layer was dried and evaporated, to give **49a**. (1.89 g, 6.86 mmol, yield 98%): ^{1}H-NMR ($CDCl_3$) δ: 0.27 (s, 9H, Me); 1.44 (s, 3H, Me); 1.48 (s, 3H, Me); 2.35 (d, 1H, *J* = 14 Hz, CH_2); 2.47 (d, 1H, *J* = 14 Hz, CH_2); 6.82-6.86 (m, 1H, Ar); 6.97-7.05 (m, 1H, Ar); 7.24-7.32 (m, 1H, Ar); 7.54-7.58 (m, 1H, Ar) ppm.

6-bromo-3,4-dihydro-2,2-dimethyl-4-[(trimethylsilyl)oxy]-2H-1-benzopyran-4-carbonitrile 49b.
Compound **49b** (2.55 g, 10 mmol) was obtained from **48b** following the procedure described for **49a**. The crude product was directly used in the next step without further purification. **49b** (3.26 g, 9.20 mmol, yield 92%): ^{1}H-NMR ($CDCl_3$) δ : 0.28 (s, 9H, Me); 1.41 (s, 3H, Me); 1.45 (s, 3H, Me); 3.28 (d, 1H, *J* = 14.1 Hz, CH_2); 2.43 (d, 1H, *J* = 14.1 Hz, CH_2); 6.71 (d, 1H, *J* = 8.8 Hz, Ar); 7.35 (dd, 1H, *J* = 8.8; 2.4 Hz, Ar); 7.61 (d, 1H, *J* = 2.4 Hz, Ar). MS (*m/z*): 353 (M^+, 61%); 284 (M^+- $Si(CH_3)_3$ 100%).

4-(aminomethyl)-2,2-dimethylchroman-4-ol 50a.
A solution of **49a** (1.38 g, 5.00 mmol) in THF was added dropwise at 0°C to a solution of $LiAlH_4$ (1M in THF, 10 mmol) The reaction mixture was stirred at rt for 1h, then quenched with water and NaOH. The resulting lithium salts were filtered and the solution was evaporated to give **50a** that was used without further purification (0.92 g, 4.40 mmol, yield 88%): ^{1}H-NMR ($CDCl_3$) δ: 1.36 (s, 3H, Me); 1.42 (s, 3H, Me); 2.01 (s, 2H, CH_2); 2.77 (d, 1H, *J* = 13 Hz, CH_2); 2.98 (d, 1H, *J* = 13 Hz, CH_2); 6.80-6.84 (m, 1H, Ar); 6.88-6.96 (m, 1H, Ar); 7.13-7.21 (m, 1H, Ar); 7.36-7.40 (m, 1H, Ar). MS (*m/z*): 207 (M^+, 25%); 177 (M^+-CH_2NH_2, 100%).

6-bromo-4-(aminomethyl)-2,2-dimethylchroman-4-ol 50b.
A solution of **49b** (1.77 g, 5.00 mmol)) in THF was added dropwise at -10°C to a solution of $LiAlH_4$ (1M in THF, 10 mmol) The reaction mixture was stirred at -10°C for 1h, then quenched with water and NaOH. The resulting lithium salts were filtered and the solution was evaporated to give **50b** that was used without further purification (1.07 g, 3.75 mmol, yield 75%): ^{1}H-NMR ($CDCl_3$) δ: 1.35 (s, 3H, Me); 1.42 (s, 3H, Me); 1.98 (s, 2H, CH_2); 2.78 (d, 1H, *J* = 12.8 Hz, CH_2); 2.96 (d, 1H, *J* = 12.8 Hz, CH_2); 6.71 (d, 1H, *J* = 8.8 Hz, Ar); 7.26 (dd, 1H, *J* = 8.8; 2.4 Hz, Ar); 7.53 (d, 1H, *J* = 2.4 Hz, Ar). MS (*m/z*): 286 (M^+, 16%); 255 (M^+- CH_2NH_2, 100%).

2-chloro-*N*-[(4-hydroxy-2,2-dimethyl-3,4-dihydro-2*H*-chromen-4-yl)methyl] acetamide 51a.
A solution of NaOH (0.19 g, 4.75 mmol) in H_2O (8,5 mL) was added to a solution of **50a** (0.83 g, 4.00 mmol) in CH_2Cl_2 (12 mL). The mixture was stirred, cooled to 0° C and treated dropwise with chloroacetyl chloride (0.73 g, 6.5 mmol). At the end of the addiction, the mixture was stirred vigorously at r.t. for 1 h. The layers were separated and the CH_2Cl_2 solution was washed with diluted HCl and H_2O, dried and evaporated to give **51a** (0.88 g, 3.13 mmol, yield 66%): ^{1}H-NMR ($CDCl_3$) δ: 1.36 (s, 3H, Me); 1.42 (s, 3H, Me); 2.02 (s, 2H, CH_2); 3.56 (dd, 1H, *J* = 13.7; 7.2 Hz, CH_2); 3.73 (dd, 1H, *J* = 13.7; 5 Hz,

CH_2); 4.08 (s, 2H, CH_2); 6.82-6.87 (m, 1H, Ar); 6.92-7.0 (m, 1H, Ar); 7.18-7.25 (m, 1H, Ar); 7.41-7.46 (m, 1H, Ar).

***N*-[(6-bromo-4-hydroxy-2,2-dimethyl-3,4-dihydro-2*H*-chromen-4-yl)methyl]-2-chloroacetamide 51b**.
Compound **51b** was obtained from **50b** ((1.68 g, 5.87 mmol) and chloroacetyl chloride (1.07 g, 9.5 mmol).following the procedure described for **50a**.The crude product was directly used in the next step without further purification. **51b** (1.36g, 3.76 mmol, yield 64%): ^{1}H-NMR ($CDCl_3$) δ: 1.34 (s, 3H, Me); 1.42 (s, 3H, Me); 1.95 (d, 1H, *J* = 14.6 Hz, CH_2); 2.04 (d, 1H, *J* = 14.6 Hz, CH_2); 3.52 (dd, 1H, *J* = 13.9; 7.1 Hz, CH_2); 3.70 (dd, 1H, *J* = 13.9; 5.1 Hz, CH_2); 4.11 (s, 2H, CH_2); 6.74 (d, 1H, *J* = 8.8 Hz, Ar); 7.30 (dd, 1H, *J* = 8.8; 2.4 Hz, Ar); 7.57 (d, 1H, *J* = 2.4 Hz, Ar).

2,2-dimethyl-2,3-dihydro-5'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-5'-one 52a. *tert*-BuOK (1.45 g, 13 mmol) was added portionwise over 1 h to a stirred solution of **51a** (2.5 mmol) in toluene (20 mL). The reaction mixture was stirred at rt for 2 h, then the solvent was evaporated. The residue was diluted with EtOAc and the organic layer washed with water, dried and evaporated to give corresponding compounds **52a** (0.45 g, 1.80 mmol, yield 72%): ^{1}H-NMR ($CDCl_3$) δ: 1.40 (s, 3H, Me); 1.43 (s, 3H, Me); 2.04 (d, 1H, *J* = 14.6 Hz, CH_2); 2.42 (d, 1H, *J* = 14.6 Hz, CH_2); 3.25 (dd, 1H, *J* = 12.4; 4.3 Hz, CH_2); 3.93 (d, 1H, *J* = 12.4 Hz, CH_2); 4.22 (d, 1H, *J* = 17.5 Hz, CH_2); 4.34 (d, 1H, *J* = 17.5 Hz,); 6.84-6.99 (m, 2H, Ar); 7.21-7.29 (m, 1H, Ar); 7.45 (dd, 1H, *J* = 7.86; 1.47 Hz, Ar). MS (*m/z*): 248 (M^+, 20%).

6-bromo-2,2-dimethyl-2,3-dihydro-5'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-5'-one 52b.
Compound **52b** was obtained from **51b** (0.75 g, 2.07 mmol) following the procedure described for **52a**.The crude product was directly used in the next step without further purification. **52b** (0.54 g, 1.65 mmol, yield 80%): ^{1}H-NMR ($CDCl_3$) δ: 1.38 (s, 3H, Me); 1.41 (s, 3H, Me); 2.03 (d, 1H, *J* = 14.6 Hz, CH_2); 2.41 (d, 1H, *J* = 14.6 Hz, CH_2); 3.23 (dd, 1H, *J* = 12.4; 4.5 Hz, CH_2); 3.88 (d, 1H, *J* = 12.4 Hz, CH_2); 4.23 (d, 1H, *J* = 17.7 Hz, CH_2); 4.36 (d, 1H, *J* = 17.7 Hz, CH_2); 6.75 (d, 1H, *J* = 8.8 Hz, Ar); 7.33 (dd, 1H, *J* = 8.8; 2.4 Hz, Ar); 7.57 (d, 1H, *J* = 2.4 Hz, Ar). MS (*m/z*) : 327 (M^+, 48%).

2,2-dimethyl-2,3-dihydrospiro[chromene-4,22-[1,4]oxazinane] 53a.
A solution of **52a** (0.7 g, 2.83 mmol)) in THF (3 mL) was added to a solution of $LiAlH_4$ (1 M in THF, 8.30 mmol) cooled at 0 °C. The mixture was refluxed for 1 h, then water and NaOH 1 M was added, and the resulting suspension was filtered. The solvent was evaporated, and the crude product was used without further purification.**53a** (0.35 g, 1.5 mmol, 53% yield): ^{1}H-NMR ($CDCl_3$) δ :

1.37 (s, 3H, Me); 1.41 (s, 3H, Me); 2.11 (d, 1H, *J* = 14.7 Hz, CH_2); 2.58 (d, 1H, *J* = 14.7 Hz, CH_2); 2.78 (d, 1H, *J* =12.2 Hz, CH_2); 2.86-3.12 (m, 2H CH_2); 3.23 (d, 1H, *J* = 12.2 Hz, CH_2); 3.65-3.73 (m, 1H, CH_2); 3.85-3.98 (m, 1H, CH_2); 6.82 (d, 1H, *J* = 8.1 Hz, Ar); 6.90-6.98 (m, 1H, Ar); 7.15-7.23 (m, 1H, Ar); 7.57 (dd, 1H, *J* = 1.7, 7.8 Hz, Ar).

6-bromo-2,2-dimethyl-2,3-dihydrospiro[chromene-4,22-[1,4]oxazinane] 53b.
A solution of **52b** (0.22 g, 0.67 mmol) in THF (3 mL) was added to a solution of BH_3 ·SMe_2 (2 M in THF 2.70 mmol). The resulting mixture was heated for 30 min by microwave irradiation at 100 °C and with a power of 150 W, and then water was added and the solvent evaporated. The aqueous phase was acidified with HCl 1 N, neutralized with NaOH 1 N and extracted with EtOAc. The organic layer was dried, and the solvent was evaporated. The crude product was transformed into the hydrochloride salt to give **53b** (0.54 g, 1.55 mmol, 88% yield): ^{1}H-NMR ($CDCl_3$) δ: 1.37 (s, 3H, Me); 1.45 (s, 3H, Me); 2.45 (d, 1H, *J* =14.7 Hz, CH_2); 2.63 (d, 1H, *J* = 14.7 Hz, CH_2); 3.20-3.42 (m, 4H, CH_2); 3.92 (d, 1H, *J* = 13.2 Hz, CH_2); 4.16-4.30 (m, 1H, CH_2); 6.74 (d, 1H, *J* = 8.6 Hz, Ar); 7.34 (dd, 1H, *J* = 2.3, 8.6 Hz, Ar); 7.51 (d, 1H, *J* = 2.3 Hz, Ar).

2,2-dimethyl-4'-(4-nitrobenzyl)-2,3-dihydro-5'*H*-spiro[chromene-4,2'-[1,4]-oxazinan]-5'-one 54a.
Compound **54a** was synthesised from **52a** (0. 62 g, 2.5 mmol). and 4-nitrobenzyl bromide (0.65 g, 3 mmol) following the procedure described for **15a**. The crude product was purified by chromatography eluting with Hexane/EtOAc (1:1) **54a** (0.39 g, 1.03 mmol, yield 41%): ^{1}H-NMR ($CDCl_3$) δ: 1.24 (s, 3H, Me); 1.33 (s, 3H, Me); 1.74 (d, 1H, *J* = 14.6 Hz, CH_2); 2.31 (d, 1H, *J* = 14.6 Hz, CH_2); 3.06 (d, 1H, *J* = 12.4 Hz, CH_2); 3.83 (d, 1H, *J* = 12.4 Hz, CH_2); 4.31 (d, 1H, *J* = 11 Hz, CH_2); 4.40 (d, 1H, *J* = 11 Hz, CH_2); 4.53 (d, 1H, *J* = 14.8 Hz, CH_2); 4.90 (d, 1H, *J* = 14.8 Hz, CH_2); 6.78-6.95 (m, 2H, Ar); 7.16-7.24 (m, 1H, Ar); 7.31-7.36 (m, 1H, Ar); 7.47 (d, 2H, *J* = 8.8 Hz, Ar); 8.20 (d, 2H, *J* = 8.8 Hz, Ar). MS (*m/z*): 382 (M^+, 60%). Anal. ($C_{21}H_{22}N_2O_5$) C, H, N.

6-bromo-2,2-dimethyl-4'-(4-nitrobenzyl)-2,3-dihydro-5'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-5'-one 54b.
Compound **54b** was synthesised from 4**9b** (0.8 g, 2.44 mmol) following the same procedure described above for the preparation of **15a**. The crude product was purified by chromatography eluting with Hexane/EtOAc (1:1) to give **54b** (0.6 g, 1.32 mmol, yield 54%): ^{1}H-NMR ($CDCl_3$) δ: 1.22 (s, 3H, Me); 1.31 (s, 3H, Me); 1.71 (d, 1H, *J* = 14.6 Hz,); 2.28 (d, 1H, *J* = 14.6 Hz, CH_2); 3.06 (d, 1H, *J* = 12.4 Hz, CH_2); 3.77 (d, 1H, *J* = 12.4 Hz, CH_2); 4.26 (d, 1H, *J* = 17.7 H CH_2); 4.40 (d, 1H, *J* = 17.7 Hz, CH_2); 4.60 (d, 1H, *J* = 14.6 Hz, CH_2); 4.81 (d,

1H, *J* = 14.6 Hz, CH_2); 6.69 (d, 1H, *J* = 8.8 Hz, Ar); 7.28 (dd, 1H, *J* = 8.8; 2.4 Hz, Ar); 7.42 (d, 1H, *J* = 2.4 Hz, Ar); 7.48 (d, 2H, *J* = 8.8 Hz, Ar); 8.2 (d, 2H, *J* = 8.8 Hz, Ar). MS (*m/z*) : 462 (M^+, 18%). Anal. ($C_{21}H_{21}BrN_2O_5$) C, H, N.

4'-(4-aminobenzyl)-2,2-dimethyl-2,3-dihydro-5'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-5'-one 5a: To a solution of compound **54a** (1.5 g, 4 mmol) in MeOH (50 mL) was added carbon (0.216 g) and $FeCl_3$. The reaction mixture was warmed to 60 °C, then hydrazine monohydrate was added dropwise (3.22 mL, 66 mmol). The mixture was refluxed overnight then filtered on a celite pad. Methanol was concentrated, the residue diluted with $CHCl_3$, dried over Na_2SO_4 and evaporated to give **5a** (1.19 g, 3.4 mmol, 85%).^{1}H-NMR ($CDCl_3$) δ: 1.14 (s, 3H); 1.31 (s, 3H); 1.71 (d, 1H, *J* = 14.6 Hz); 2.22 (d, 1H, *J* = 14.6 Hz); 3.06 (d, 1H, *J* = 12.6 Hz); 3.74 (d, 1H, *J* = 12.6 Hz); 4.11 (d, 1H, *J* = 14 Hz); 4.25 (d, 1H, *J* = 17.5 Hz); 4.35 (d, 1H, *J* = 17.5 Hz); 4.90 (d, 1H, *J* = 14 Hz); 6.63 (d, 2H, *J* = 8.4 Hz); 6.77-6.95 (m, 2H); 7.07 (d, 2H, *J* = 8.4 Hz); 7.16-7.21 (m, 1H); 7.34-7.38 (m, 1H). MS (*m/z*): 352 (M^+, 29%). Anal. ($C_{21}H_{24}N_2O_3$) C, H, N.

4'-(4-aminobenzyl)-6-bromo-2,2-dimethyl-2,3-dihydro-5'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-5'-one 5b: Compound **5b** was synthesized from **54b** (0.14 g, 0.3 mmol) following the same procedure described above for the preparation of **5a**. **5b** (yield 87%, 0.11 g, 0.26 mmol): ^{1}H-NMR (DMSO) δ: 1.10 (s, 3H); 1.23 (s, 3H); 1.64 (d, 1H, *J* = 14.6 Hz); 2.30 (d, 1H, *J* = 14.6 Hz); 3.03 (d, 1H, *J* = 12.6 Hz); 3.78 (d, 1H, *J* = 12.8 Hz); 4.08 (d, 1H, *J* = 14 Hz); 4.22 (s, 2H); 4.63 (d, 1H, *J* = 14 Hz); 6.52 (d, 2H, *J* = 8.2 Hz); 6.80-6.87 (m, 1H); 6.95 (d, 2H, *J* = 8 Hz); 7.36 (dd, 1H, *J* = 8.4; 2,4 Hz); 7.57 (d, 1H, *J* = 2.4 Hz). MS (*m/z*): 432 (M^+, 15%). Anal. ($C_{21}H_{23}BrN_2O_3$) C, H, N.

4'-(N-(4-acetamidobenzyl))-2,2-dimethyl-2,3-dihydro-5'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-5'-one 1a: Compound **1a** was obtained from **5a** (0.18 g, 0.514 mmol) in acetone (5 mL) and K_2CO_3 (0.106 g, 0.76 mmol) and acetic anhydride (0.04 mL, 0.514 mmol) following the same procedure described above for **31a**. The crude product was purified by tritration with Et_2O to yield **1a** (0.17 g, 0.45 mmol, 88%) mp: 85-88 °C; ^{1}H-NMR ($CDCl_3$) δ: 1.18 (s, 3H); 1.32 (s, 3H); 1.73 (d, 1H, *J* = 14.6 Hz); 2.17 (s, 3H, $COCH_3$); 2.26 (d, 1H, *J* = 14.6 Hz); 3.06 (d, 1H, *J* = 12.6 Hz); 3.78 (d, 1H, *J* = 12.6 Hz); 4.23-4.44 (m, 3H); 4.88 (d, 1H, *J* = 14.2 Hz); 6.78-6.94 (m, 2H); 7.16-7.26 (m, 3H); 7.33-7.37 (m, 1H); 7.48 (d, 2H, *J* = 8.42 Hz). ^{13}C-NMR ($CDCl_3$) δ : 168.50; 154.86; 136.47; 130.42; 130.18; 127.42; 120.92; 120.21; 118.39; 74.26; 69.22; 65.64; 63.78; 55.62; 45.10; 40.60; 39.60; 29.22; 28.10. MS (*m/z*): 394 (M^+, 32%). Anal. ($C_{23}H_{26}N_2O_4$) C, H, N.

4'-(N-(4-methansolfonamido)benzyl)-6-bromo-2,2-dimethyl-2,3-dihydro-5'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-5'-one 2b: Compound **2b** was obtained from **5b** (0.5 g, 1.15 mmol) and methanesulfonyl chloride (0.115 mL, 1.5 mmol) following the same procedure described for **32a**. The crude product was purified by was purified by cromathography eluting with Hexane/EtOAc (3:7) to give **2b** (0.275 g , 0.54 mmol, 47%).: mp.: 68-70°C; ^{1}H-NMR (DMSO) δ: 1.13 (s, 3H); 1.24 (s, 3H); 1.68 (d, 1H, *J* = 14.6 Hz); 2.35 (d, 1H, *J* = 14.6 Hz); 2.96 (s, 3H); 3.09 (d, 1H, *J* = 12.8 Hz); 3.90 (d, 1H, *J* = 12.8 Hz); 4.25 (s, 2H); 4.32 (d, 1H, *J* = 14.6 Hz); 4.67 (d, 1H, *J* = 14.6 Hz); 6.74 (d, 1H, *J* = 8.8 Hz); 7.17 (d, 2H, *J* = 8.4 Hz); 7.29 (d, 2H, *J* = 8.4 Hz); 7.36 (dd, 1H, *J* = 8.8; 2.4 Hz); 7.59 (d, 1H, *J* = 2.4 Hz). ^{13}C-NMR ($CDCl_3$) δ: 218.54; 205.47; 165.94; 152.30; 136.28; 132.67; 132.33; 129.63; 129.45; 123.20; 120.35; 119.62; 112.22; 74.19; 68.45; 63.03; 54.92; 48.82; 39.92; 28.41; 25.44. MS (*m/z*): 510 (M^+, 35%). Anal. ($C_{22}H_{25}BrN_2O_5S$) C, H, N.

ethyl(2,2-dimethyl-5'-oxo-2,3-dihydro-4'H-spiro[chromene-4,2'-[1,4] oxazinan] -4'-yl) acetate 55a.
Compound **55a** was obtained from **52a** (0.6 g, 2.42 mmol) and ethyl bromoacetate (0.48 g, 2.90 mmol) following the same procedure described for **15a**. The crude product was used for the next step without further purification. **55a** (0.26 g, 0.77 mmol, yield 32%): ^{1}H-NMR ($CDCl_3$) δ : 1.21- 1.41 (m, 9H, Me); 2.21 (d, 1H, *J* = 14.7 Hz, CH_2); 2.42 (d, 1H, *J* = 14.7 Hz, CH_2); 3.22 (d, 1H, *J* = 12.08 Hz, CH_2); 3.93- 4.41 (m, 7H); 6.84 (dd, 1H, *J* = 1.1; 8.2 Hz, Ar); 6.90- 6.98 (m, 1H, Ar); 7.19- 7.28 (m, 1H, Ar); 7.49 (dd, 1H, *J* = 1.6; 7.8 Hz, Ar) ppm.

ethyl(6-bromo-2,2-dimethyl-5'-oxo-2,3-dihydro-4'H-spiro[chromene-4,2'-[1,4] oxazinan]-4'-yl)acetate 55b.
Compound **55b** was obtained from **52b** (0.33 g, 1.00 mmol) and ethyl bromoacetate (0.20 g, 1.20 mmol) following the same procedure described for **15a**. The crude product was used for the next step without further purification. **55b** (0.18 g, 0.44 mmol, yield 44%) ^{1}H-NMR ($CDCl_3$) δ : 1.20- 1.41 (m, 9H, Me); 2.20-2.45 (m, 2H CH_2); 3.20 (d, 1H, *J* = 12.2 Hz, CH_2); 3.90- 4.44 (m, 7H CH_2); 6.73 (d, 1H, *J* = 8.6 Hz, Ar); 7.32 (dd, 1H, *J* = 2.4, 8.8 Hz, Ar); 7.61 (d, 1H, *J* = 2.6 Hz, Ar) ppm.

ethyl(2,2-dimethyl-2,3-dihydro-4'*H*-spiro[chromene-4,2'-[1,4]oxazinan]-4'-yl)acetate 56a.
Compound **56a** was obtained from **53a** (0.45 g, 1.92 mmol) and ethyl bromoacetate (0.32 g, 1.92 mmol) following the same procedure described for **18a**. The crude product was used for the next step without further purification. **56a** (0.55 g, 1.90 mmol, yield 99%): ^{1}H-NMR ($CDCl_3$) δ : 1.22- 1.33 (m, 3H, Me); 1.36 (s, 3H, Me); 1.41 (s, 3H, Me); 2.28 (d, 1H, *J* = 14.8 Hz, CH_2); 2.56

(d, 1H, *J* = 14.8 Hz, CH_2); 2.65-2.90 (m, 2H, CH_2); 3.19 (d, 1H, *J* = 16.7 Hz, CH_2); 3.37 (d, 1H, *J* = 16.7 Hz, CH_2); 3.73-3.82 (m, 3H, CH_2); 4.01-4.28 (m, 3H, CH_2); 6.78-6.96 (m, 2H, Ar); 7.14-7.23 (m, 1H, Ar); 7.56 (dd, 1H, *J* = 1.5, 7.8 Hz, Ar) ppm.

2,2-dimethyl-2,3-dihydro-2'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-2'-one 57a.

A solution of **50a** (1.31 g, 6.33 mmol) in THF was added dropwise to a solution of *N*-*N*'-carbonyl diimidazole (CDI) (1.02 g, 6.33 mmol) in THF at 0 ˚C. The reaction mixtures were stirred rt for 5h. The solvent was evaporated under vacuum and the residue diluted with EtOAc and washed with HCl 1N and K_2CO_{3sat}. The organic layers were dried and concentrated under vacuum. The crude product was directly used for the next reaction without further purification. **57a** (1.6 g, 4.93 mmol, yield 78%): ^{1}H-NMR ($CDCl_3$) δ : 1.42 (s, 6H, Me); 2.17 (d, 1H, *J* = 14.6 Hz, CH_2); 2.46 (d, 1H, *J* = 14.6 Hz, CH_2); 3.63 (d, 1H, *J* = 8.8 Hz, CH_2); 3.89 (d, 1H, *J* = 8.9 Hz, CH_2); 6.83 (d, 1H, *J* = 8.2 Hz, Ar); 6.93-7.01 (m, 1H, Ar); 7.22-7.29 (m, 1H, Ar); 7.49 (dd, 1H, *J* = 1.3, 7.8 Hz, Ar) ppm.

6-bromo-2,2-dimethyl-2,3-dihydro-2'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-2'-one 57b.

Compound **57b** was obtained from **50b** (1.81 g, 6.33 mmol) following the procedure described for **57a**.The crude product was was directly used for the next reaction without further purification. **57b** (2.15 g, 5.31 mmol, yield 84%): ^{1}H-NMR ($CDCl_3$) δ : 1.41 (s, 6H, Me); 2.14 (d, 1H, *J* =14.6 Hz, CH_2); 2.42 (d, 1H, *J* =14.6 Hz, CH_2); 3.64 (d, 1H, *J* = 9.0 Hz, CH_2); 3.86 (d, 1H, *J* = 8.9 Hz, CH_2); 6.72 (d, 1H, *J* =8.8 Hz, Ar); 7.33 (dd, 1H, *J* =8.8, 2.4 Hz, Ar); 7.58 (d,1H, *J* =2.4 Hz, Ar) ppm.

ethyl(2,2-dimethyl-2'-oxo-2,3-dihydro-3'H-spiro[chromene-4,5'-[1,3] oxazolidin]-3'-yl)acetate 58a.

Compound **58a** was obtained from **57a** (0.44 g, 1.37 mmol) and ethyl bromoacetate (0.23g, 1.37 mmol) following the same procedure described for **15a**. The crude product was directly used for the next reaction without further purification. **58a** (0.23 g, 0.56 mmol, yield 41%): ^{1}H-NMR ($CDCl_3$) δ : 1.33 (t, 3H, *J* =7.1 Hz, Me); 1.42 (s, 6H, Me); 2.17 (d, 1H, *J* =14.6 Hz, CH_2); 2.46 (d, 1H, *J* =14.6 Hz, CH_2); 3.64 (d, 1H, *J* =8.4 Hz, CH_2); 3.98 (d, 1H, *J* =8.4 Hz, CH_2); 4.02 (d, 1H, *J* =18.0 Hz, CH_2); 4.20 (d, 1H, *J* =18.0 Hz, CH_2); 4.25 (q, 2H, *J* =7.1 Hz, CH_2); 6.82 (d, 1H, *J* =8.2 Hz, Ar); 6.94-7.02 (m, 1H, Ar); 7.20-7.29 (m, 1H, Ar); 7.58 (dd, 1H, *J* =1.6, 7.8 Hz, Ar) ppm.

ethyl(6-bromo-2,2-dimethyl-2'-oxo-2,3-dihydro-3'H-spiro[chromene-4,5'-[1,3] oxazolidin]-3'-yl)acetate 58b.
Compound **58b** was obtained from **57b** (0.55 g, 1.37 mmol) and ethyl bromoacetate (0.23g, 1.37 mmol) following the same procedure described for **15a**. The crude product was directly used for the next reaction without further purification. **58b** (0.33 g, 0.67 mmol, yield 49%): ^{1}H-NMR ($CDCl_3$) δ : 1.32 (t, 3H, *J* = 7.1 Hz, Me); 1.41 (s, 6H, Me); 2.16 (d, 1H, *J* = 14.7 Hz, CH_2); 2.45 (d, 1H, *J* = 14.7 Hz, CH_2); 3.67 (d, 1H , *J* = 8.7 Hz, CH_2); 3.93 (d, 1H, *J* = 8.7 Hz, CH_2); 4.01-4.33 (m, 4H, CH_2); 6.7 (d, 1H, *J* = 8.7 Hz, Ar); 7.33 (dd , 1H, *J* = 2.4, 8.7 Hz, Ar); 7.68 (d, 1H, *J* = 2.38 Hz, Ar) ppm.

2-(4-methoxyphenyl)-2-methyl-2,3-dihydro-4H-chromen-4-one 59a.
To a solution of 2-hydroxy acetophenone (1.00 g, 7.35 mmol) in CH_3CN (10 mL) was added 4-methoxy-acetophenone (1.1 g, 7.35 mmoli) and pirrolidine (0.49 g; 7.35 mmoli). The mixture was stirred at rt for 1h, then refluxed for 48 h. The solvent was removed under reduced pressure and the residue diluted with EtOAc and washed with HCl 1N, NaOH 1N and water. The organic layers were dried and evaporated. The crude product was purified by flash column chromatography eluted with Hex/EtOAc 7/3 to give **59a** (0.37 g, 1.4 mmol, yield 19%)
^{1}H-NMR ($CDCl_3$) δ : 1.74 (s, 3H, Me); 3.08 (d, 1H, *J* = 16.4 Hz, CH_2); 3.31 (d, 1H, *J* = 16.4 Hz, CH_2); 3,74 (s, OMe); 6.8 (d, 2H, *J* = 8.7 Hz, Ar); 6.88 - 6.95(m, 1H, Ar); 7.03 (d, 1H, *J* = 8.2 Hz, Ar) 7.32 (d, 2H, *J* = 8.7 Hz, Ar); 7.41-7.49 (m, 1H, Ar); 7.76 (dd, 1H, *J* = 1.6, 7.7 Hz, Ar) ppm.

6-bromo-2-(4-methoxyphenyl)-2-methyl-2,3-dihydro-4H-chromen-4-one 59b.
Compound **59b** was obtained from 5-bromo-2-hydroxy acetophenone (1.58 g, 7.35 mmol) and 4-methoxy-acetophenone (1.1 g, 7.35 mmoli) following the procedure described for **59a**. The crude product was purified by flash column chromatography eluted with Hex/EtOAc 7/3 to give **59b** (0.56 g, 1.62 mmol, yield 22%): ^{1}H-NMR ($CDCl_3$) δ : 1.74 (s, 3H, Me); 3.04 (d, 1H, *J* = 16.5 Hz, CH_2); 3.3 (d, 1H, *J* = 16.5 Hz, CH_2); 3.75 (s, OMe); 6.81 (d, 2H, *J* = 8.8 Hz, Ar); 6.92 (d, 1H, *J* = 8.8 Hz,; Ar); 7.3 (d, 2H, *J* = 8.8 Hz, Ar); 7.51 (dd, 1H, *J* = 2.6; 8.8 Hz, Ar); 7.85 (d, 1H, *J* = 2.6 Hz, Ar) ppm.

2-(4-chlorophenyl)-2-methyl-2,3-dihydro-4*H*-chromen-4-one 60a.
Compound **60a** was obtained from 2-hydroxy acetophenone (5.00 g, 36.72 mmol) and 4-chloro-acetophenone (5.68 g, 36.72 mmol) following the procedure described for **59a**. The crude product was purified by flash column chromatography eluted with Hex/EtOAc (95:5) to give **60a** (4.00 g, 14.66 mmol, yield 40%): mp 82-85°C. ^{1}H-NMR ($CDCl_3$) δ : 1.74 (s, 3H, Me); 3.08 (d, 1H, *J* = 16.5 Hz, CH_2); 3.27 (d, 1H, *J* = 16.5 Hz, CH_2); 6.91-6.99 (m, 1H,

Ar); 7.05 (d, 1H, *J* = 8.2 Hz, Ar); 7.26 (d, 2H, *J* = 8.8 Hz, Ar); 7.35 (d, 2H, *J* = 8.8 Hz, Ar); 7.44-7.52 (m, 1H, Ar); 7.76 (dd, 1H, *J* = 1.6, 7.9 Hz, Ar) ppm.

6-bromo-2-(4-chlorophenyl)-2-methyl-2,3-dihydro-4*H*-chromen-4-one 60b.
Compound **60b** was obtained from 5-bromo-2-hydroxy acetophenone (7.89 g, 36.72 mmol) and 4-chloro-acetophenone (5.68 g, 36.72 mmol) following the procedure described for **59a**. The crude product was used for the next step without further purification. **60b** (5.81 g, 16.52 mmol, yield 45 %): mp 90-92°C. ^{1}H-NMR ($CDCl_3$) δ : 1.74 (s, 3H, Me); 3.07 (d, 1H, *J* = 16.6 Hz, CH_2); 3.28 (d, 1H, *J* = 16.6 Hz, CH_2); 6.95 (d, 1H, *J* = 8.8 Hz, Ar); 7.24-7.34 (m, 4H, Ar); 7.54 (dd, 1H, *J* = 2.6, 8.8 Hz, Ar); 7.86 (d, 1H, *J* = 2.6 Hz, Ar) ppm.

4-[(trimethylsilyl)oxy]-2-(4-methoxyphenyl)-2-methyl-3,4-dihydro-2H-chromene-4-carbonitrile 61a.
Compound **61a** was obtained from **59a** (0.9 g, 3.35 mmol) following the procedure described for **49a**. The crude product was directly used in the next step without further purification. **61a** (0.97 g, 2.65 mmol, yield 79%): ^{1}H-NMR ($CDCl_3$) δ : 0.27 (s, 9H, Me); 1.71 (s, 3H, Me); 2.56 (d, 1H, *J* = 14.3 Hz, CH_2); 2.79 (d, 1H, *J* = 14.3 Hz, CH_2); 3.78 (s, OMe); 6.87 (d, 2H, *J* = 8.7 Hz, Ar); 6.97 - 7.04 (m, 2H, Ar); 7.25 - 7.36 (m, 3H, Ar); 7.52 (dd, 1H, *J* = 1.8, 8.0 Hz, Ar) ppm.

6-bromo-4-[(trimethylsilyl)oxy]-2-(4-methoxyphenyl)-2-methyl-3,4-dihydro-2H-chromene-4-carbonitrile 61b.
Compound **61b** was obtained from **59b** (1.1 g, 3.35 mmol) following the procedure described for **49a**. The crude product was directly used in the next step without further purification. **61b** (1.34 g, 3.01 mmol, yield 90%): ^{1}H-NMR ($CDCl_3$) δ : 0.28 (m, 9H, Me); 1.70 (s, 3H, Me); 2.53 (d, 1H, *J* = 14.1 Hz, CH_2); 2.81 (d, 1H, *J* = 14.1 Hz, CH_2); 3.79 (s, OMe); 6.852- 6.93 (m, 3H, Ar); 7.252-7.30 (m, 3H, Ar); 7.38- 7.44 (m, 1H, Ar); 7.58- 7.59 (m, 1H, Ar) ppm.

4-[(trimethylsilyl)oxy]-2-(4-chlorophenyl)-2-methyl-3,4-dihydro-2H-chromene -4-carbonitrile 62a.
Compound **62a** was obtained from **60a** (0.53 g, 1.95 mmol) following the procedure described for **49a**. The crude product was directly used in the next step without further purification. **62a** (0.27g, 0.72 mmol, yield 37 %): ^{1}H-NMR ($CDCl_3$) δ : 0.28 (s, 9H, Me); 1.72 (s, 3H, Me); 2.61 (d, 1H, *J* = 14.1 Hz, CH_2); 2.77 (d, 1H, *J* = 14.1 Hz, CH_2); 6.99-7.07 (m, 2H, Ar); 7.27-7.39 (m, 5H, Ar); 7.52 (dd, 1H, *J* = 1.6, 8.1 Hz, Ar) ppm.

6-bromo-4-[(trimethylsilyl)oxy] -2-(4-chlorophenyl)-2-methyl-3,4-dihydro-2H-chromene-4-carbonitrile 62b.
Compound **62b** was obtained from **60b** (0.68 g, 1.95 mmol) following the procedure described for **49a**. The crude product was directly used in the next step without further purification.**62b** (0.74 g, 1.64 mmol, yield 84 %) mp 81-84°C: ^{1}H-NMR ($CDCl_3$) δ : 0.30 (s, 9H, Me); 1.70(s, 3H, Me); 2.55 (d, 1H, *J* = 14.3 Hz, CH_2); 2.78 (d, 1H, *J* = 14.3 Hz, CH_2); 6.92 (d, 1H, *J* = 8.8 Hz, Ar); 7.28-7.36 (m, 4H, Ar); 7.42 (dd, 1H, *J* = 2.1, 8.8 Hz, Ar); 7.59 (d, 1H, *J* = 2.1 Hz, Ar) ppm.

4-(aminomethyl)-2-(4-methoxyphenyl)-2-methyl-3,4-dihydro-2*H*-chromen-4-ol 63a.
Compound **63a** was obtained from **61a** (0.97 g, 2.65 mmol) following the procedure described for **50a**.The crude product was directly used in the next step without further purification. **63a** (0.75 g, 2.5 mmol, yield 94%): ^{1}H-NMR ($CDCl_3$) δ : 1.68 (s, 3H, Me); 2.10 (d, 1H, *J* = 13.3 Hz, CH_2); 2.24 (d, 1H, *J* = 14.1 Hz, CH_2); 2.49 (d, 1H, *J* = 13.3 Hz, CH_2); 2.56 (d, 1H, *J* = 14.1 Hz, CH_2); 3.78 (s, OMe); 6.81 - 7.03 (m, 4H, Ar); 7.19 - 7.41 (m, 4H, Ar) ppm.

4-(aminomethyl)-6-bromo-2-(4-methoxyphenyl)-2-methyl-3,4-dihydro-2*H*-chromen-4-ol 63b.
Compound **63b** was obtained from **61b** (1.34 g, 3.01 mmol) following the procedure described for **50a**.The crude product was directly used in the next step without further purification. **63b** (0.85 g, 2.26 mmol, yield 75%): ^{1}H-NMR ($CDCl_3$) δ : 1.64 (s, 3H, Me); 2.02 (d, 1H, *J* = 13.2 Hz, CH_2); 2.19 (d, 1H, *J* = 14.2 Hz, CH_2); 2.38 (d, 1H, *J* = 13.2 Hz, CH_2); 2.53 (d, 1H, *J* = 14.2 Hz, CH_2); 3.76 (s, OMe); 6.80- 6.90 (m, 3H, Ar); 7.21- 7.32 (m, 3H, Ar); 7.48 (d, 1H, *J* = 2.4 Hz, Ar) ppm.

4-(aminomethyl)-2-(4-chlorophenyl)-2-methyl-3,4-dihydro-2*H*-chromen-4-ol 64a.
Compound **64a** was obtained from **62a** (0.26 g, 0.71 mmol) following the procedure described for **50a**.The crude product was directly used in the next step without further purification. **64a** (0.21 g, 0.71 mmol, yield 100%): ^{1}H-NMR ($CDCl_3$) δ : 1.67 (s, 3H, Me); 2.13 (d, 1H, *J* = 13.2 Hz, CH_2); 2.27 (d, 1H, *J* = 14.1 Hz, CH_2); 2.41-2.57 (m, 2H, CH_2); 6.92-7.07 (m, 2H, Ar); 7.22-7.41 (m, 6H, Ar) ppm.

4-(aminomethyl)-6-bromo-2-(4-chlorophenyl)-2-methyl-3,4-dihydro-2H-chromen-4-ol 64b.
Compound **64b** was obtained from **62b** (0.32 g, 0.71 mmol) following the procedure described for **50a**.The crude product was directly used in the next step without further purification. **64b** (0.24 g, 0.64 mmol, yield 90%): ^{1}H-NMR

($CDCl_3$) δ : 1.66 (s, 3H, Me); 2.11 (d, 1H, *J* = 13.2 Hz, CH_2); 2.25 (d, 1H, *J* = 14.3 Hz, CH_2); 2.46 (d, 1H, *J* = 13.2 Hz, CH_2); 2.51 (d, 1H, *J* = 14.3 Hz, CH_2); 6.89 (d, 1H, *J* = 8.7 Hz, Ar); 7.25-7.37 (m, 5H, Ar); 7.51 (d, 1H, *J* = 2.2 Hz, Ar) ppm.

2-(4-methoxyphenyl)-2-methyl-2,3-dihydro-2'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-2'-one 65a.
Compound **65a** was obtained from **63a** (0.23 g, 0.78 mmol) following the procedure described for **57a**.The crude product was directly used in the next step without further purification. **65a** (0.06 g, 0.19 mmol, yield 25%): ^{1}H-NMR ($CDCl_3$) δ : 1.65 (s, 3H, Me); 2.37 (Br-d, 2H, *J* = 14.5 Hz, CH_2); 2,69-2.94 (m, 2H, CH_2); 3.75 (s, 3H, OMe); 6.73- 7.07 (m, 4H, Ar); 7.213- 7.42 (m, 4H, Ar) ppm.

6-bromo-2-(4-methoxyphenyl)-2-methyl-2,3-dihydro-2'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-2'-one 65b.
Compound **65b** was obtained from **63b** (0.29 g, 0.78 mmol) following the procedure described for **57a**.The crude product was directly used in the next step without further purification. **65b** (0.23 g, 0.58 mmol, yield 75%): ^{1}H-NMR ($CDCl_3$) δ : 1.73 (s, 3H, Me); 2.63- 2.65 (m, 2H, CH_2); 3.06- 3.20 (m, 2H, CH_2); 3.79 (s, 3H, OMe); 6.85 (d, 2H, *J* = 8.9 Hz, Ar); 6.901-6.95 (m, 1H, Ar); 7.21-7.26 (m, 3H, Ar); 7.4 (dd, 1H, *J* = 2.4; 8.8 Hz, Ar); 7.51- 7.52 (m, 1H, Ar) ppm.

2-(4-chlorophenyl)-2-methyl-2,3-dihydro-2'H-spiro[chromene-4,5'-[1,3]oxazolidin]-2'-one 66a.
Compound **66a** was obtained from **64a** (0.21 g, 0.71 mmol) following the procedure described for **57a**.The crude product was directly used in the next step without further purification. **66a** (0.23 g, 0.71 mmol, yield 100 %): ^{1}H-NMR ($CDCl_3$) δ: 1.73 (s, 3H, Me); 2.55 (d, 1H, *J* = 14.3 Hz, CH_2); 2.72 (d, 1H, *J* = 14.3 Hz, CH_2); 3.18 (d, 1H, *J* = 9.2 Hz, CH_2); 3.36 (d, 1H, *J* = 9.2 Hz, CH_2); 6.92-7.46 (m, 8H, Ar) ppm.

6-bromo-2-(4-chlorophenyl)-2-methyl-2,3-dihydro-2'*H*-spiro[chromene-4,5'-[1,3]oxazolidin]-2'-one 66b.
Compound **66b** was obtained from **64b** (0.24 g, 0.64 mmol) following the procedure described for **57a**.The crude product was directly used in the next step without further purification. **66b** (0.23 g, 0.56 mmol, yield 88 %): mp 45-48°C. ^{1}H-NMR ($CDCl_3$) δ : 1.72 (s, 3H, Me); 2.54 (d, 1H, *J* = 14.5 Hz, CH_2); 2.69 (d, 1H, *J* = 14.5 Hz, CH_2); 3.16 (d, 1H, *J* = 9.2 Hz, CH_2); 3.31 (d, 1H, *J* = 9.2 Hz, CH_2); 6.92 (d, 1H, *J* = 8.8 Hz, Ar); 7.25-7.44 (m, 5H, Ar); 7.53 (d, 1H, *J* = 2.2 Hz, Ar) ppm.

ethyl[2-(4-methoxyphenyl)-2-methyl-2'-oxo-2,3-dihydro-3'H-spiro [chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetate 67a.
Compound **67a** was obtained from **65a** (0.06 g, 0.19 mmol) following the procedure described for **58a**.The crude product was directly used in the next step without further purification. **67a** (0.03 g, 0.07 mmol, yield 38%): ^{1}H-NMR ($CDCl_3$) δ : 1.20- 1.32 (m, 3H, Me); 1.66 (s, 3H, Me); 2.04- 2.32 (m, 2H, CH_2); 2.51-2.83 (m, 2H CH_2); 3.77 (s, OMe); 4.08-4.24 (m, 2H, CH_2); 6.75- 7.05 (m, 4H, Ar); 7.19-7.43 (m, 4H, Ar) ppm.

ethyl[6-bromo-2-(4-methoxyphenyl)-2-methyl-2'-oxo-2,3-dihydro-3'H-spiro [chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetate 67b.
Compound **67b** was obtained from **65b** (0.23 g, 0.58 mmol) following the procedure described for **58a**.The crude product was directly used in the next step without further purification. **67b** (0.25 g, 0.51 mmol, yield 88 %): ^{1}H-NMR ($CDCl_3$) δ : 1.21- 1.35 (m, 3H, Me); 1.72 (s, 3H, Me); 2.67 (s, 2H, CH_2); 3.16 (d, 1H, *J* = 9 Hz, CH_2); 3.29 (d, 1H, *J* = 9 Hz, CH_2); 3.79 (s, 3H, OMe); 3.97 (s, 2H, CH_2); 4.18-4.29 (m, 2H, CH_2); 6.82- 6.94 (m, 3H, Ar); 7.22- 7.26 (m, 3H); 7.4 (dd, 1H, *J* = 2.4; 8.6 Hz, Ar); 7.58 (d, 1H, *J* = 2.4 Hz, Ar) ppm.

ethyl[2-(4-chlorophenyl)-2-methyl-2'-oxo-2,3-dihydro-3'H-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetate 68a.
A solution of **66a** (0.49 g, 1.50 mmol) in dry THF was added dropwise, at -78 ˚C, under N_2 atmosphere to a solution of *n*-BuLi (1.05 ml, 1.70 mmol, 1.6 M in Hexane) and the reaction mixtures were stirred for 1h. Ethyl bromoacetate (0.25 g, 1.50 mmol) was added dropwise at -78 ˚C and the resulting mixtures were allowed to warm to rt and stirred overnight. The mixtures were quenched with NH_4Cl_{sat} and then the solvent was evaporated. The residues were extracted with EtOAc, the combined organic layers were dried and concentrated under vacuum.The crude product was directly used in the next step without further purification. **68a** (0.39 g, 0.94 mmol, yield 63 %): ^{1}H-NMR ($CDCl_3$) δ : 1.20-1.37 (m, 3H, Me); 1.73 (s, 3H, Me); 2.61 (d, 1H, *J* = 14.5 Hz, CH_2); 2.73 (d, 1H, *J* = 14.5 Hz, CH_2); 3.24 (d, 1H, *J* = 8.9 Hz, CH_2); 3.49 (d, 1H, *J* = 8.9 Hz, CH_2); 4.01 (d, 2H, *J* = 2.4 Hz, CH_2); 4.22 (q, 2H, *J* = 7.2 Hz, CH_2); 6.99-7.06 (m, 2H, Ar); 7.27-7.39 (m, 5H, Ar); 7.50-7.55 (m, 1H, Ar) ppm.

ethyl[6-bromo-2-(4-chlorophenyl)-2-methyl-2'-oxo-2,3-dihydro-3'H-spiro [chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetate 68b.
Compound **68b** was obtained from **66b** (0.61 g, 1.50 mmol) following the procedure described for **66a**.The crude product was directly used in the next step without further purification. **68b** (0.56 g, 1.14 mmol, yield 76 %): ^{1}H-NMR ($CDCl_3$) δ : 1.21-1.34 (m, 3H, Me); 1.73 (s, 3H, CH3); 2.40-2.73 (m, 2H, CH_2); 3.12-3.52 (m, 2H, CH_2); 4.01 (s, 2H, CH_2); 4.10-4.30 (m, 2H, CH_2); 6.90-7.05 (m, 1H, Ar); 7.25-7.45 (m, 5H, Ar); 7.54 (d, 1H, *J* = 2.4 Hz, Ar) ppm.

{[4-hydroxy-2-(4-methoxyphenyl)-2-methyl-3,4-dihydro-2H-chromen-4-yl] methyl}carbamic chloride 69a.
Compound **69a** was obtained from **63a** (1.6 g, 5.35 mmol) and chloroacetyl chloride (0.86 g, 7.49 mmol) following the procedure described for **51a**.The crude product was directly used in the next step without further purification. **69a** (1.30 g, 3.47 mmol, yield 65 %): ^{1}H-NMR ($CDCl_3$) δ : 1.67 (s, 3H, Me); 2.29 (d, 1H, *J* = 14.2 Hz, CH_2); 2.63 (d, 1H, *J* = 14.2 Hz, CH_2); 3.45 (dd, 1H, *J* = 5.8, 14.4 Hz, CH_2); 3.74 (d, 1H, *J* = 14.6 Hz, CH_2); 3.75 (s,OMe); 3.99 (d,1H, *J* = 15.5 Hz, CH_2); 4.09 (d, 1H, *J* = 15.5 Hz, CH_2); 6.79-6.90 (m, 3H, Ar); 6.94-7.021 (m,1H, Ar); 7.18 -7.36 (m, 3H, Ar); 7.45 (d, 1H, *J* = 7.6 Hz, Ar) ppm.

{[6-bromo-4-hydroxy-2-(4-methoxyphenyl)-2-methyl-3,4-dihydro-2H-chromen -4-yl]methyl}carbamic chloride 69b.
Compound **69b** was obtained from **63b** (0.8 g, 2.11 mmol) and chloroacetyl chloride (0.34 g, 2.954 mmol) following the procedure described for **51a**.The crude product was directly used in the next step without further purification. **69b** (0.8 g, 1.76 mmol, yield 47%). ^{1}H-NMR ($CDCl_3$) δ : 1.69 (s, 3H, Me); 2.24-2.36 (m, 2H, CH_2); 2.66 (d, 1H, *J* = 14.2 Hz, CH_2); 3.38-3.48 (m, 1H, CH_2); 3.78 (s, 3H, OMe); 4.03 (d,1H, *J* = 15.7 Hz, CH_2); 4.13 (d, 1H, *J* = 15.7 Hz, CH_2); 6.80-6.94 (m, 3H, Ar); 7.24-7.38 (m, 3H, Ar); 7.61 (d, 1H, *J* = 2.6 Hz, Ar) ppm.

2-(4-methoxyphenyl)-2-methyl-2,3-dihydro-2'H-spiro[chromene-4,5'-[1,3] oxazinan]-2'-one 70a.
Compound **70a** was obtained from **69a** (1.30 g, 3.47 mmol) following the procedure described for **52a**.The crude product was directly used in the next step without further purification. **70a** (0.73 g, 2.16 mmol, yield 63 %). ^{1}H-NMR ($CDCl_3$) δ : 1.72 (s, 3H, Me); 2.24 (d, 1H, *J* = 12.8 Hz, CH_2); 2.33 (d, 1H, *J* = 13.7 Hz, CH_2); 3.01 (d, 1H, *J* = 13.7 Hz, CH_2); 3.33 (d, 1H, *J* = 12.8 Hz, CH_2); 3.76 (s, OMe); 4.38 (s, 2H CH_2); 6.80 (d, 2H, *J* = 7.6 Hz, Ar); 6.92 – 6.99 (m, 1H, Ar); 7.03 - 7.07 (m, 1H, Ar); 7.15 -7.39 (m, 5H, Ar) ppm.

6-bromo-2-(4-methoxyphenyl)-2-methyl-2,3-dihydro-2'H-spiro[chromene-4,5'-[1,3]oxazinan]-2'-one 70b.
Compound **70b** was obtained from **69b** (0.8 g, 1.76 mmol) following the procedure described for **52a**.The crude product was directly used in the next step without further purification. **70b** (0.63 g, 1.5 mmol, yield 85%). ^{1}H-NMR ($CDCl_3$) δ: 1.74 (s, 3H, Me); 2.29 (d, 1H, *J* = 12.8 Hz, CH_2); 2.35 (d, 1H, *J* = 13.7 Hz, CH_2); 3.05 (d, 1H, *J* = 13.7 Hz, CH_2); 3.40 (d, 1H, *J* = 12.8 Hz, CH_2); 3.78 (s, OMe); 4.40 (s, 2H CH_2); 6.82 (d, 2H, *J* = 8.8 Hz, Ar); 7.03 (d, 1H, *J* =

8.6 Hz, Ar); 7.29-7.35 (m, 2H, Ar); 7.45-7.50 (m, 1H, Ar); 7.55 (d, 1H, *J* = 2.4 Hz, Ar) ppm.

ethyl(2,-(4-methoxyphenyl)-2-methyl-2'-oxo-2,3-dihydro-3'H-spiro[chromene-4,5'-[1,3]oxazinan]-3'-yl)acetate 71a.
Compound **71a** was obtained from **70a** (0.73 g, 2.16 mmol) and ethylbromoacetate (0.78 g, 2.16 mmol) following the procedure described for **15a**.The crude product was directly used in the next step without further purification. **71a** (0.47 g, 1.10 mmol, yield 51 %): ^{1}H-NMR ($CDCl_3$) δ: 1.26 (t, 3H, *J* = 7.1 Hz, Me); 1.73 (s, 3H); 2.21 (d, 1H, *J* = 12.4 Hz, CH_2); 2.39 (d, 1H, *J* = 13.5 Hz, CH_2); 3.04 (d, 1H, *J* = 13.5 Hz, CH_2); 3.18 (d, 1H, *J* = 17.3 Hz, CH_2); 3.64 (d, 1H, *J* = 12.6 Hz, CH_2); 3.78 (s, OMe); 4.16 (q, 2H, *J* = 7.1 Hz, CH_2); 4.34 (d, 1H, *J* = 17.3 Hz, CH_2); 4.41 (s, 2H CH_2); 6.82 (d, 2H, *J* = 8.7 Hz, Ar); 6.93 - 7.08 (m, 2H, Ar); 7.21 – 7.36 (m, 3H, Ar); 7.44 (dd, 1H, *J* = 7.7, 1.4 Hz, Ar) ppm.

ethyl(6-bromo-2-(4-methoxyphenyl)-2-methyl -2'-oxo-2,3-dihydro-3'H-spiro [chromene-4,5'-[1,3]oxazolidin]-3'-yl)acetate 71b.
Compound **71b** was obtained from **70b** (0.3 g, 0.72 mmol) and ethylbromoacetate (0.12 g, 0.72 mmol) following the procedure described for **15a**.The crude product was directly used in the next step without further purification. **71b** (0.22 g, 0.4 mmol, yield 55%).

(2E)-1-(2-hydroxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one 72a.
To a solution of 4-methoxybenzaldehyde (1.51 g, 11.09 mmol) in absolute EtOH (20 mL) was added 2-hydroxyacetophenone (1.50 g, 11.09 mmol). The reaction mixture was stirred for 5 min and then NaOH (1.33 g, 33.29 mmol) was added. The resulting mixture was stirred for 5h rt and then HCl 1N was added. The precipitate was filtered to give **72a** directly used in the next reaction without further purification. **72a** (2.75 g, 10.87 mmol, yield 99%) ^{1}H-NMR ($CDCl_3$) δ : 3.87 (s, 3H, OMe); 6.90-7.05 (m, 2H, Ar); 6.96 (d, 2H, *J* = 8.8 Hz, CH); 7.45-7.54 (m, 1H, Ar); 7.54 (d, 1H, *J* = 15.3 Hz, CH); 7.64 (d, 2H, *J* = 8.8 Hz, Ar); 7.87-7.94 (m, 1H, CH); 7.91 (d, 1H, *J* = 15.3 Hz, CH) ppm.

(2E)-1-(5-bromo-2-hydroxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one 72b.
Compound **72b** was obtained from 5-bromo-2-hydroxyacetophenone (1.50 g, 6.97 mmol) and 4-methoxybenzaldehyde (0.95 g, 6.97 mmol) following the same procedure described for **72a**. The crude product was directly used in the next reaction without further purification. **72b** (2.3 g, 6.9 mmol, yield 99%) ^{1}H-NMR ($CDCl_3$) δ: 3.87 (s, 3H, OMe); 6.89-6.90 (m, 3H, Ar); 7.43 (d, 1H, *J* = 15.3 Hz, CH); 7.55 (dd, 1H, *J* = 2.4, 8.8 Hz, Ar); 7.60 (d, 2H, *J* = 8.8 Hz, Ar); 7.90 (d, 1H, *J* = 15.3 Hz, CH); 8.00 (d, 1H, *J* = 2.4 Hz, Ar) ppm.

(2E)-3-(4-chlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one 73a.
Compound **73a** was obtained from 2-hydroxyacetophenone (0.63 g, 4.65 mmol) and 4-chlorobenzaldehyde (0.65 g, 4.65 mmol) following the same procedure described for **72a** and used for the next reaction without further purification. **73a** (1.18 g, 4.56 mmol, yield 98%): ^{1}H-NMR ($CDCl_3$) δ: 6.91-7.05 (m, 2H, Ar); 7.40 (d, 2H, *J* = 8.6 Hz, Ar); 7.47-7.65 (m, 4H, Ar, CH); 7.82-7.92 (m, 2H, Ar, CH) ppm.

(2E)-1-(5-bromo-2-hydroxyphenyl)-3-(4-chlorophenyl)prop-2-en-1-one 73b.
Compound **73b** was obtained from 5-bromo-2-hydroxyacetophenone (1.00 g, 4.65) and 4-chlorobenzaldehyde (0.65 g, 4.65 mmol) following the same procedure described for **72a** and used for the next reaction without further purification. **73b** (1.38 g, 4.09 mmol, yield 88%): ^{1}H-NMR ($CDCl_3$) δ : 6.95 (d, 1H, *J* = 9.0 Hz, Ar); 7.41-7.49 (m, 2H, Ar); 7.55-7.65 (m, 4H, Ar, CH); 7.89 (d, 1H, *J* = 15.6 Hz, CH); 7.99 (d, 1H, *J* = 2.4 Hz, Ar) ppm.

2-(4-methoxyphenyl)-2,3-dihydro-4H-chromen-4-one 74a.
The solution of **72a** (2.75 g, 10.87 mmol) in glacial AcOH (20 mL) was refluxed for 72h. After cooling to rt the reaction mixture was poured in H_2O and extracted with EtOAc. The organic layer was dried and concentrated under vacuum to give **74a** without further purification (1.13 g, 4.46 mmol, yield 41%) ^{1}H-NMR ($CDCl_3$) δ : 2.86 (dd, 1H, *J* = 3.1, 16.8 Hz, CH_2); 3.12 (dd, 1H, *J* = 13, 16.8 Hz, CH_2); 3.84 (s, 3H, OMe); 5.44 (dd, 1H, *J* = 3.1, 13 Hz, CH); 6.94-7.09 (m, 4H, Ar); 7.38-7.55 (m, 4H, Ar); 7.93 (dd, 1H, *J* = 1.7, 8.1 Hz, Ar) ppm.

6-bromo-2-(4-methoxyphenyl)-2,3-dihydro-4H-chromen-4-one 74b.
Compound **74b** was obtained from **72b** (2.3 g, 6.9 mmol) following the procedure described for **74a**. The crude product was used for the next reaction without further purification. **74b** (1.15 g, 3.45 mmol, yield 50 %): ^{1}H-NMR ($CDCl_3$) δ : 2.87 (dd, 1H, *J* = 3.2, 16.8 Hz, CH_2); 3.10 (dd, 1H, *J* = 12.8, 16.8 Hz, CH_2); 3.84 (s, 3H, OMe); 5.42 (dd, 1H, *J* = 3.2, 12.8 Hz, CH); 6.92-6.99 (m, 3H, Ar); 7.57 (dd, 1H, *J* = 2.6, 8.6 Hz, Ar); 7.66 (d, 2H, *J* = 9.0 Hz, Ar); 8.03 (d, 1H, *J* = 2.6 Hz, Ar) ppm.

2-(4-chlorophenyl)-2,3-dihydro-4H-chromen-4-one 75a.
Compound **75a** was obtained from **73a** (1.18 g, 4.56 mmol) following the procedure described for **74a**.The crude product was directly used for the next reaction without further purification. **75a** (0.28 g, 1.09 mmol, yield 24%): ^{1}H-NMR ($CDCl_3$) δ : 2.88 (dd, 1H, *J* = 3.5, 16.9 Hz, CH_2); 3.05 (dd, 1H, *J* = 12.6,

16.9 Hz, CH_2); 5.47 (dd, 1H, *J* = 3.5, 12.6 Hz, CH); 6.91-7.11 (m, 2H, Ar); 7.40-7.67 (m, 5H, Ar); 7.84-7.96 (m, 1H, Ar) ppm.

6-bromo-2-(4-chlorophenyl)-2,3-dihydro-4H-chromen-4-one 75b.
Compound **75b** was obtained from **73b** (1.38 g, 4.09 mmol) purification following the procedure described for **74a**.The crude product was directly used for the next reaction without further purification. **75b** (1.05 g, 3.1 mmol, yield 76%): ^{1}H-NMR ($CDCl_3$) δ : 2.88 (dd, 1H, *J* = 3.7, 17.2 Hz, CH_2); 3.04 (dd, 1H, *J* = 12.4, 17.2 Hz, CH_2); 5.45 (dd, 1H, *J* = 3.7, 12.4 Hz, CH); 6.96 (d, 1H, *J* = 8.8 Hz, Ar); 7.38-7.44 (m, 4H, Ar); 7.59 (dd, 1H, *J* = 2.5, 8.8 Hz, Ar); 8.03 (d, 1H, *J* = 2.5 Hz, Ar) ppm.

2-(2,4-dichlorophenyl)-2,3-dihydro-4H-chromen-4-one 76a.
To a solution of 2,4 dichlorobenzaldheyde (2.57 g, 14.68 mmoli) in MeOH was added 2-hydroxy acetophenone (2.00 g, 14.68 mmoli) and KOH (0.33 g, 5.87 mmoli). The reaction mixture was refluxed for 7h. The solvent was evaporated under reduced pressure and the curde product was purified by flash column chromatography eluted with Hex/EtOAc (9/1) to give **76a** (4.00 g, 13.8 mmol, yield 94%): ^{1}H-NMR ($CDCl_3$) δ : 2.76-3.10 (m, 2H, CH_2); 5.82 (dd, 1H, *J* = 13.0, 3.1 Hz, CH); 7.05-7.13 (m, 1H, Ar); 7.31-7.58 (m, 3H, Ar); 7.68-7.73 (m, 1H, Ar); 7.93-7.98 (m, 1H, Ar) ppm.

6-bromo-2-(2,4-dichlorophenyl)-2,3-dihydro-4H-chromen-4-one 76b.
Compound **76b** was obtained from 5-bromo-2hydroxyacetophenone (2.00 g, 9.30 mmol) and 2,4 dichlorobenzaldheyde (1.63 g; 9.30 mmoli) following the procedure described for **76a**. The crude product was directly used for the next reaction without further purification. **76b** (2.2 g, 5.95 mmol, yield 64%): mp 133-135°C. ^{1}H-NMR ($CDCl_3$) δ : 2.82 (dd, 1H, *J* = 13.2, 17.0 Hz, CH_2); 3.03 (dd, 1H, *J* = 3.0, 17.0 Hz, CH_2); 5.80 (dd, 1H, *J* = 3.0, 13.2 Hz, CH); 6.98 (d, 1H, *J* = 8.8 Hz, Ar); 7.38 (dd, 1H, *J* = 1.9, 8.4 Hz, Ar); 7.45 (d, 1H, *J* = 1.9 Hz, Ar); 7.61 (dd, 1H, *J* = 2.6, 8.8 Hz, Ar); 7.67 (d, 1H, *J* = 8.4 Hz, Ar); 8.06 (d, 1H, *J* = 2.6 Hz, Ar) ppm.

6-bromo-2-(2,4-difluorophenyl)-2,3-dihydro-4H-chromen-4-one 77b.
Compound **77b** was obtained from 5-bromo-2hydroxyacetophenone (1.00 g, 4.65 mmol) and 2,4-difluorobenzaldehyde (0.66 g; 4.65 mmoli) following the synthestic procedure described above for **76a**. The crude product was purified by crystallizzation from EtOH to give **77b** (0.38 g, 1.12 mmol, yield 24%): ^{1}H-NMR ($CDCl_3$) δ : 2.85-3.12 (m, 2H, CH_2); 5.71 (dd, 1H, *J* = 12.4, 3.7 Hz, CH); 6.83-7.02 (m, 3H, Ar); 7.53-7.65 (m, 2H, Ar); 8.05 (d, 1H, *J* = 2.4 Hz, Ar) ppm.

6-bromo-2-(4-bromo-2-fluorophenyl)-2,3-dihydro-4H-chromen-4-one 78b.
Compound **78b** was synthesised from 5-bromo-2hydroxyacetophenone (1.00 g, 4.65) and 4-bromo-2-fluorobenzaldehyde (0.94 g, 4.65 mmol) following the synthetic procedure described for **76a** The crude product was purified by flash column chromatography eluted with Hex/EtOAc (9/1) to give **78b** (0.65 g, 1.62 mmol, yield 35%): ^{1}H-NMR ($CDCl_3$) δ : 2.87-3.09 (m, 2H, CH_2); 5.70 (dd, 1H, *J* = 4.8, 11.3 Hz, CH); 6.96 (d, 1H, *J* = 8.8 Hz, Ar); 7.29-7.57 (m, 3H, Ar); 7.60 (dd, 1H, *J* = 2.5, 8.8 Hz, Ar); 8.05 (d, 1H, *J* = 2.5 Hz, Ar) ppm.

4-[(trimethylsilyl)oxy]-2-(4-methoxyphenyl)-3,4-dihydro-2H-chromene-4-carbonitrile 79a.
Compound **79a** was obtained from **74a** (1.13 g, 4.46 mmol) following the procedure described for **49a**. The crude product was purified by flash column chromatography eluted with Hex/EtOAc (9/1) to give **79a** (0.17 g, 0.48 mmol, yield 10%): ^{1}H-NMR ($CDCl_3$) δ : 0.30 (s, 9H, Me); 2.42-2.68 (m, 2H, CH_2); 3.84 (s, 3H, OMe); 5.30-5.37 (m, 1H, CH); 6.87-7.07 (m, 2H, Ar); 6.97 (d, 2H, *J* = 8.7 Hz, Ar); 7.23-7.32 (m, 1H, Ar); 7.40 (d, 2H, *J* = 8.7 Hz, Ar); 7.57 (dd, 1H, *J* = 1.6, 7.9 Hz, Ar) ppm.

6-bromo-4-[(trimethylsilyl)oxy]-2-(4-methoxyphenyl)-3,4-dihydro-2H-chromene-4 -carbonitrile 79b.
Compound **79b** was obtained from **74b** (1.15 g, 3.45 mmol) following the procedure described for **49a**. The crude product was directly used in the next step without further purification. **79b** (1.15 g, 2.65 mmol, yield 77 %): ^{1}H-NMR ($CDCl_3$) δ : 0.32 (s, 9H, Me); 2.34-2.66 (m, 2H, CH_2); 3.81 (s, 3H, OMe); 5.38 (dd, 1H, *J* = 3.4, 12.9 Hz, CH); 6.85-7.05 (m, 3H, Ar); 7.26-7.43 (m, 3H, Ar); 7.65-7.72 (m, 1H, Ar) ppm.

2-(4-chlorophenyl)-4-[(trimethylsilyl)oxy]-3,4-dihydro-2H-chromene-4-carbo nitrile 80a.
Compound **80a** was obtained from **75a** (0.37 g, 1.30 mmol) following the procedure described for **49a**. The crude product was used in the next reaction without further purification. **80a** (0.39 g, 1.1 mmol, yield 84 %): ^{1}H-NMR ($CDCl_3$) δ : 0.29 (s, 9H, Me); 2.36-2.67 (m, 2H, CH_2); 5.35 (m 1H, CH); 6.89-6.93 (m, 1H, Ar); 7.01-7.09 (m, 1H, Ar); 7.30-7.42 (m, 5H, Ar); 7.65-7.72 (dd, 1H, *J* = 1.5, 7.8 Hz, Ar) ppm.

6-bromo-2-(4-chlorophenyl)-4-[(trimethylsilyl)oxy]-3,4-dihydro-2H-chromene-4-carbonitrile 80b.
Compound **80b** was obtained from **75b** (1.05 g, 3.1 mmol) following the procedure described for **49a**. The crude product was purified by flash column chromatography eluted with $CHCl_3$/Hex (1/1) to give **80b** (0.58 g, 1.33 mmol, yield 43 %): ^{1}H-NMR ($CDCl_3$) δ : 0.31 (s, 9H, Me); 2.32-2.44 (m, 1H, CH_2);

2.62 (dd, 1H, *J* = 2.0, 13.4 Hz, CH_2); 5.36 (dd, 1H, *J* =2.0, 11.8 Hz, CH); 6.80 (d, 1H, *J* = 8.8 Hz, Ar); 7.35-7.47 (m, 5H, Ar); 7.65 (d, 1H, *J* = 2.4 Hz, Ar) ppm.

2-(2,4-dichlorophenyl)-4-[(trimethylsilyl)oxy]-3,4-dihydro-2H-chromene-4-carbonitrile 81a.
Compound **81a** was obtained from **76a** (0.56 g, 1.91 mmoli) following the procedure described for **49a**. The crude product was directly used in the next step without further purification. **81a** (0.52 g, 1.32 mmoli, yield 69%): ^{1}H-NMR ($CDCl_3$) δ : 0.21 (s, 9H, Me); 2.22 (dd, 1H, *J* = 13.6, 11.8 Hz, CH_2); 2.70 (dd, 1H, *J* = 13.6, 1.9 Hz, CH_2); 5.70 (dd, 1H, *J* = 1.9, 11.8 Hz, CH); 6.98 (dd, 1H, *J* = 1.1, 8.3 Hz, Ar); 7.07 (dt, 1H, *J* = 1.1, 7.6 Hz, Ar); 7.32-7.46 (m, 3H, Ar); 7.59-7.65 (m, 1H, Ar) ppm.

6-bromo-2-(2,4-dichlorophenyl)-4-[(trimethylsilyl)oxy]-3,4-dihydro-2H-chromene-4-carbonitrile 81b.
Compound **81b** was obtained from **76b** (2.2 g, 5.95 mmol) following the procedure described for **49a**. The crude product was directly used in the next step without further purification. **81b** (1.68 g, 3.57 mmol, yield 60%) mp139-142°C: ^{1}H-NMR ($CDCl_3$) δ : 0.31 (s, 9H, Me); 2.17 (dd, 1H, *J* = 11.7, 13.4 Hz, CH_2); 2.82 (dd, 1H, *J* = 1.7, 13.4 Hz, CH_2); 5.72 (dd, 1H, *J* = 1.7, 11.7 Hz, CH); 6.84 (d, 1H, *J* = 8.8 Hz, Ar); 7.33-7.38 (m, 1H, Ar); 7.40 (dd, 1H, *J* = 2.4, 8.8 Hz, Ar); 7.46 (d, 1H, *J* = 2.0 Hz, Ar); 7.57 (d, 1H, *J* = 8.4 Hz, Ar); 7.66 (d, 1H, *J* = 2.4 Hz, Ar) ppm.

6-bromo-2-(2,4-difluorophenyl)-4-[(trimethylsilyl)oxy]-3,4-dihydro-2H-chromene-4-carbonitrile 82b.
Compound **82b** was obtained from **77b** (0.38 g, 1.12 mmol) following the procedure described for **49a**. The crude product was directly used in the next step without further purification. **82b** (0.23 g, 0.54 mmol, yield 48%): ^{1}H-NMR ($CDCl_3$) δ : 0.31 (s, 9H, Me); 2.43 (dd, 1H, *J* = 13.3, 11.9 Hz, CH_2); 2.68 (dd, 1H, *J* = 2.0, 13.3 Hz, CH_2); 5.62 (dd, 1H, *J* = 2.0, 11.9 Hz, CH); 6.80 (d, 1H, *J* = 8.8 Hz, Ar); 6.84-7.01 (m, 2H, Ar); 7.39 (dd, 1H, *J* = 2.4, 8.8 Hz, Ar); 7.45-7.57 (m, 1H, Ar); 7.65 (d, 1H, *J* = 2.4 Hz, Ar) ppm.

6-bromo-2-(4-bromo-2-fluorophenyl)- 4-[(trimethylsilyl)oxy] -3,4-dihydro-2H-chromene-4-carbonitrile 83b.
Compound **83b** was obtained from **78b** (0.45 g, 1.12 mmol) following the procedure described for **49a**. The crude product was directly used in the next step without further purification yield **83b** (0.5 g, 1.00 mmol, 89%): ^{1}H-NMR ($CDCl_3$) δ : 0.31 (s, 9H, Me); 2.40 (dd, 1H, *J* = 13.3, 11.7 Hz, CH_2); 2.68 (dd, 1H, *J* = 1.7, 13.3 Hz, CH_2); 5.61 (dd, 1H, *J* = 1.7, 11.7 Hz, CH); 6.81 (d, 1H, *J* = 8.8 Hz, Ar); 7.30-7.42 (m, 4H, Ar); 7.64 (d, 1H, *J* = 2.4 Hz, Ar) ppm.

4-(aminomethyl)-2-(4-methoxyphenyl)-3,4-dihydro-2H-chromen-4-ol 84a.
Compound **84a** was obtained from **79a** (0.48 g, 1.17 mmol)following the procedure described for **50a**. The crude product was directly used in the next step without further purification. **84a** (0.25 g, 0.89 mmol, yield 76%) ^{1}H-NMR ($CDCl_3$) δ : 2.15-2.33 (m, 2H, CH_2); 3.02 (d, 1H *J* = 13.2 Hz, CH_2); 3.11 (d, 1H, *J* = 13.2 Hz, CH_2); 3.83 (s, 3H, OMe); 5.09 (dd, 1H, *J* = 4.2, 10.4 Hz, CH); 6.73-7.08 (m, 4H, Ar); 7.15-7.23 (m, 1H, Ar); 7.38 (d, 2H, *J* = 8.6 Hz, Ar); 7.47-7.50 (m, 1H, Ar) ppm.

4-(aminomethyl)-6-bromo-2-(4-methoxyphenyl)-3,4-dihydro-2H-chromen-4-ol 84b.
Compound **84b** was obtained from **79b** (1.15 g, 2.65 mmol) following the procedure described for **50a**.The crude product was directly used in the next step without further purification. **84b** (0.41 g, 1.14 mmol, yield 43 %): ^{1}H-NMR ($CDCl_3$) δ : 1.90-2.55 (m, 2H, CH_2); 3.53-3.94 (m, 5H, CH_2, OMe); 4.96-5.13 (m, 1H, CH); 6.71-6.96 (m, 3H, Ar); 7.03-7.39 (m, 3H, Ar); 7.54-7.64 (m, 1H, Ar) ppm.

4-(aminomethyl)-2-(4-chlorophenyl)-3,4-dihydro-2H-chromen-4-ol 85a.
Compound **85a** was obtained from **80a** (0.39 g, 1.10 mmol) following the procedure described for **50a**.The crude product was directly used in the next step without further purification. **85a** (0.31 g, 1.09 mmol, yield 99%). ^{1}H-NMR ($CDCl_3$) δ : 2.06-2.37 (m, 2H, CH_2); 2.89-3.25 (m, 2H, CH_2); 5.14 (dd, 1H, *J* = 2.6, 12.3 Hz, CH); 6.85-7.02 (m, 2H, Ar); 7.16-7.25 (m, 1H, Ar); 7.33-7.41 (m, 4H, Ar); 7.49 (dd, 1H, *J* = 1.7, 7.8 Hz, Ar) ppm.

4-(aminomethyl)-6-bromo-2-(4-chlorophenyl)-3,4-dihydro-2H-chromen-4-ol 85b.
Compound **85b** was obtained from **80b** (0.58 g, 1.33 mmol) following the procedure described for **50a**.The crude product was directly used in the next step without further purification. **85b** (0.4 g, 1.08 mmol, yield 81 %). ^{1}H-NMR ($CDCl_3$) δ : 2.01-2.35 (m, 2H, CH_2); 2.89-3.13 (m, 2H, CH_2); 5.08-5.17 (m, 1H, CH); 6.75 (d, 1H, *J* = 8.6 Hz, Ar); 7.16-7.50 (m, 5H, Ar); 7.60-7.62 (m, 1H, Ar) ppm.

4-(aminomethyl)-2-(2,4-dichlorophenyl)-3,4-dihydro-2H-chromen-4-ol 86a.
Compound **86a** was obtained from **81a** (0.52 g, 1.32 mmoli) following the procedure described for **50a**.The crude product was directly used in the next step without further purification. **86a** (0.32 g, 1.00 mmol, yield 76%): ^{1}H-NMR ($CDCl_3$) δ : 1.78-2.47 (m, 2H, CH_2); 2.96-3.35 (m, 2H, CH_2); 5.45-5.52 (m, 1H, CH); 6.88-7.04 (m, 2H, Ar); 7.18-7.66 (m, 4H, Ar) ppm.

4-(aminomethyl)-6-bromo-2-(2,4-dichlorophenyl)-3,4-dihydro-2H-chromen-4-ol 86b.
Compound **86b** was obtained from **81b** (1.68 g, 3.57 mmol) following the procedure described for **50a**.The crude product was directly used in the next step without further purification. **86b** (1.25 g, 3.1 mmol, yield 87 %): mp 75-77°C. ^{1}H-NMR ($CDCl_3$) δ : 1.81-1.94 (m, 1H, CH_2); 2.41 (dd, 1H, *J* = 1.8, 13.7 Hz, CH_2); 2.94 (d, 1H, *J* = 13.4 Hz, CH_2); 3.23 (d, 1H, *J* = 13.4 Hz, CH_2); 5.46 (dd, 1H, *J* = 1.8, 12.8 Hz, CH); 6.79 (d, 1H, *J* = 8.8 Hz, Ar); 7.29 (dd, 1H, *J* = 2.4, 8.8 Hz, Ar); 7.36-7.41 (m, 2H, Ar); 7.58-7.62 (m, 2H, Ar) ppm.

4-(aminomethyl)-6-bromo-2-(2,4-difluorophenyl)-3,4-dihydro-2H-chromen-4-ol 87b.
Compound **87b** was obtained from **82b** (0.23 g, 0.54 mmol) following the procedure described for **50a**.The crude product was directly used in the next step without further purification. **87b** (0.13 g, 0.36 mmol, yield 67%): ^{1}H-NMR ($CDCl_3$) δ : 2.10-2.56 (m, 2H, CH_2); 2.93-3.20 (m, 2H, CH_2); 5.34-5.47 (m, 1H, CH); 6.77 (d, 1H, *J* = 8.6 Hz, Ar); 6.85-7.02 (m, 2H, Ar); 7.17-7.30 (m, 1H, Ar); 7.39-7.64 (m, 2H, Ar) ppm.

4-(aminomethyl)-6-bromo-2-(4-bromo-2-fluorophenyl)-3,4-dihydro-2H-chromen-4-ol 88b.
Compound **88b** was obtained from **83b** (0.27 g, 0.54 mmol) following the procedure described for **50a**.The crude product was directly used in the next step without further purification. **88b** (0.18 g, 0.42 mmol, yield 77%): ^{1}H-NMR ($CDCl_3$) δ : 1.98-2.40 (m, 2H, CH_2); 2.92-3.21 (m, 2H, CH_2); 5.37-5.50 (m, 1H, CH); 6.77 (dd, 1H, *J* = 8.6 , 2.6 Hz, Ar); 6.90-7.62 (m, 5H, Ar) ppm.

2-(4-methoxyphenyl)-2,3-dihydro-2'H-spiro[chromene-4,5'-[1,3]oxazolidin]-2'-one 89a.
Compound **89a** was obtained from **84a** (0.25 g, 0.89 mmol) following the procedure described for **57a**.The crude product was purified by flash column chromatography eluted by Hex/EtOAc (2/3) to give **89a** (0.04 g, 0.13 mmol, yield 15%) ^{1}H-NMR ($CDCl_3$) δ : 2.41-2.69 (m, 2H, CH_2); 3.73-3.88 (m, 5H, CH_2, OMe); 5.00-5.07 (m, 1H, CH); 6.86-7.07 (m, 4H Ar); 7.23-7.30 (m, 1H, Ar); 7.39 (d, 2H, *J* =8.7 Hz, Ar); 7.44-7.48 (m, 1H, Ar) ppm.

6-bromo-2-(4-methoxyphenyl)-2,3-dihydro-2'H-spiro[chromene-4,5'-[1,3]oxazolidin]-2'-one 89b.
Compound **89b** was obtained from **84b** (0.41 g, 1.14 mmol) following the procedure described for **57a**.The crude product was directly used in the next step without further purification. **89b** (0.41 g, 1.05 mmol, yield 92 %): ^{1}H-NMR ($CDCl_3$) δ : 2.04-2.65 (m, 2H, CH_2); 3.68-3.87 (m, 5H, CH_2, OMe); 5.00-

5.13 (m, 1H, CH); 6.64-6.96 (m, 3H Ar); 7.03-7.39 (m, 3H, Ar) 7.54-7.64 (m, 1H, Ar) ppm.

2-(4-chlorophenyl)-2,3-dihydro-2'H-spiro[chromene-4,5'-[1,3]oxazolidin]-2'-one 90a.
Compound **90a** was obtained from **85a** (0.31 g, 1.09 mmol) following the procedure described for **57a**.The crude product product was directly used in the next step without further purification. **90a** (0.35 g, 1.03 mmol, yield 95%): ^{1}H-NMR ($CDCl_3$) δ : 2.36-2.57 (m, 2H, CH_2); 3.73 (d, 1H, *J* = 9 Hz, CH_2); 3.82 (d, 1H, *J* = 9 Hz, CH_2); 5.04 (dd, 1H, *J* = 3.6, 10.9 Hz, CH); 6.91 (dd, 1H, *J* = 0.9, 8.2 Hz, Ar); 6.98-7.07 (m, 1H, Ar); 7.23-7.32 (m, 1H, Ar); 7.37-7.45 (m, 4H, Ar) ppm.

6-bromo-2-(4-chlorophenyl)-2,3-dihydro-2'H-spiro[chromene-4,5'-[1,3] oxazolidin]-2'-one 90b.
Compound **90b** was obtained from **85b** (0.4 g, 1.08 mmol) following the procedure described for **57a**.The crude product was directly used in the next step without further purification. **90b** (0.31 g, 0.79 mmol, yield 73 %): ^{1}H-NMR ($CDCl_3$) δ : 2.41-2.62 (m, 2H, CH_2); 3.73-3.89 (m, 2H, CH_2); 5.01-5.11 (m, 1H, CH); 6.81 (d, 1H, *J* = 8.8 Hz, Ar); 7.29-7.49 (m, 5H, Ar); 7.58 (d, 1H, *J* = 2.3 Hz, Ar) ppm.

2-(2,4-dichlorophenyl)-2,3-dihydro-2'H-spiro[chromene-4,5'-[1,3]oxazolidin]-2'-one 91a.
Compound **91a** was obtained from **86a** (0.2 g, 0.62 mmoli) following the procedure described for **57a**.The crude product was directly used in the next step without further purification. **91a** (0.17 g, 0.5 mmol, yield: 80%): ^{1}H-NMR ($CDCl_3$) δ : 2.23-2.39 (m, 1H, CH_2); 2.58-269 (m, 1H, CH_2); 3.85-3.95 (m, 2H, CH_2); 5.40-5.54 (m, 1H, CH); 6.93-6.99 (m, 1H, Ar); 7.02-7.11 (m, 1H, Ar); 7.25-7.50 (m, 3H, Ar); 7.65-7.76 (m, 1H, Ar) ppm.

6-bromo-2-(2,4-dichlorophenyl)-2,3-dihydro-2'H-spiro[chromene-4,5'-[1,3] oxazolidin]-2'-one 91b.
Compound **91b** was obtained from **86b** (1.25 g, 3.1 mmol) following the procedure described for **57a**.The crude product was purified by crystallization from EtOH to give **91b** (0.58 g, 1.36 mmol, yield 44 %): mp 216-219°C. ^{1}H-NMR ($CDCl_3$) δ : 2.20-2.33 (m, 1H, CH_2); 2.61 (dd, 1H, *J* = 1.4, 13.6 Hz, CH_2); 3.87 (s, 2H, CH_2); 5.41 (dd, 1H, *J* = 1.4, 12.3 Hz, CH); 6.85 (d, 1H, *J* = 8.9 Hz, Ar); 7.35-7.44 (m, 3H, Ar); 7.60 (d, 1H, *J* = 2.4 Hz, Ar); 7.63 (d, 1H, *J* = 8.4 Hz, Ar) ppm.

6-bromo-2-(2,4-difluorophenyl)-2,3-dihydro-2'H-spiro[chromene-4,5'-[1,3] oxazolidin]-2'-one 92b.
Compound **92b** was obtained from **87b** (0.43 g, 1.18 mmol) following the procedure described for **57a**.The crude product was directly used in the next step without further purification. **92b** (0.31g, 0.79 mmol, yield 66%): ^{1}H-NMR ($CDCl_3$) δ : 2.45-2.54 (m, 2H, CH_2); 3.79 (d, 1H, J = 8.9 Hz, CH_2); 3.86 (d, 1H, J = 8.9 Hz, CH_2); 5.26-5.45 (m, 1H, CH); 6.74-7.14 (m, 3H, Ar); 7.21-7.67 (m, 3H, Ar) ppm.

6-bromo-2-(4-bromo-2-fluorophenyl)-2,3-dihydro-2'H-spiro[chromene-4,5'-[1,3]oxazolidin]-2'-one 93b.
Compound **93b** was obtained from **88b** (0.5 g, 1.18 mmol) following the procedure described for **57a**.The crude product was purified by crystallization from EtOH to give **93b** (0.41 g, 0.91 mmol, yield 77%): ^{1}H-NMR ($CDCl_3$) δ : 2.31-2.56 (m, 2H, CH_2); 3.79 (d, 1H, J = 9.1 Hz, CH_2); 3.86 (d, 1H, J = 9.1 Hz, CH_2); 5.35 (dd, 1H, J = 10.7, 3.3 Hz, CH); 5.79 (br s, 1H, NH); 6.82 (d, 1H, J = 8.8 Hz, Ar); 7.28-7.53 (m, 4H, Ar); 7.59 (d, 1H, J = 2.4 Hz, Ar) ppm.

ethyl[2-(4-methoxyphenyl)-2'-oxo-2,3-dihydro-3'H-spiro[chromene-4,5'-[1,3] oxazolidin]-3'-yl]acetate 94a.
Compound **94a** was obtained from **89a** (0. 48 g, 1.00 mmol) and ethylbromoacetate (0.17 g, 1.00 mol) following the procedure described for **67a**.The crude product product was directly used in the next step without further purification. **94a** (0.3 g, 0.63 mmol, yield 63%)^{1}H-NMR ($CDCl_3$) δ : 1.22-1.33 (m, 3H, Me); 2.47-2.69 (m, 2H, CH_2); 3.78-3.94 (m, 5H, CH_2, OMe); 4.06-4.29 (m, 4H, CH_2); 4.99-5.07 (m, 1H, CH); 6.88-7.07 (m, 4H, Ar); 7.23-7.30 (m, 1H, Ar); 7.39 (d, 2H, J = 8.4 Hz, Ar); 7.49-7.53 (m, 1H, Ar) ppm.

ethyl[6-bromo-2-(4-methoxyphenyl)-2'-oxo-2,3-dihydro-3'H-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetate 94b.
Compound **94b** was obtained from **89b** (0.41 g, 1.05 mmol) and ethylbromoacetate (0.17 g, 1.05 mmol) following the procedure described for **67a**.The crude product was directly used in the next step without further purification. **94b** (0.17 g, 0.37 mmol, yield 35%): ^{1}H-NMR ($CDCl_3$) δ : 1.25-1.58 (m, 3H, Me); 2.83-2.86 (m, 2H, CH_2); 3.79-3.89 (m, 5H, CH_2, OMe); 3.97-4.26 (m, 4H, CH_2); 5.46-5.50 (m, 1H, CH); 6.88-6.96 (m, 3H, Ar); 7.51-7.74 (m, 3H, Ar) 7.92-7.97 (m, 1H, Ar) ppm.

ethyl[2-(4-chlorophenyl)-2'-oxo-2,3-dihydro-3'H-spiro[chromene-4,5'-[1,3] oxazolidin]-3'-yl]acetate 95a.
Compound **95a** was obtained from **90a** (0.35 g, 1.03 mmol) and ethylbromoacetate (0.17 g, 1.03 mmol) following the procedure described for **67a**.The crude product was purified by flash column chromatography eluted

with Hex/EtOAc (3/2) to give **95a** (0.05 g, 0.11 mmol, yield 10%): ^{1}H-NMR ($CDCl_3$) δ: 1.30 (t, 3H, *J* = 7.1 Hz, Me); 2.45-2.62 (m, 2H, CH_2); 3.83 (d, 1H, *J* = 8.5 Hz, CH_2); 3.91 (d, 1H, *J* = 8.5 Hz, CH_2); 4.05 (d, 1H, *J* = 18.1 Hz, CH_2); 4.15(d, 1H, *J* = 18.1 Hz, CH_2); 4.24 (q, 2H, *J* = 7.1 Hz, CH_2); 5.00-5.15 (m, 1H, CH); 6.90-6.94 (m, 1H, Ar); 7.00-7.09 (m, 1H, Ar); 7.24-7.33 (m, 1H, Ar); 7.41 (s, 4H, Ar); 7.51(dd, 1H, *J* = 1.5, 7.8 Hz, Ar) ppm.

ethyl[6-bromo-2-(4-chlorophenyl)-2'-oxo-2,3-dihydro-3'H-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetate 95b.
Compound **95b** was obtained from **90b** (0.31 g, 0.79 mmol) and ethylbromoacetate (0.13 g, 0.79 mmol) following the procedure described for **67a**.The crude product was purified by flash column chromatography eluted with Hex/EtOAc 8/2 to give **95b** (0.32 g, 0.68 mmol, yield 86%): ^{1}H-NMR ($CDCl_3$) δ: 1.22-1.38 (m, 3H, Me); 2.24-2.62 (m, 2H, CH_2); 3.73-4.02 (m, 2H, CH_2); 4.05-4.30 (m, 4H, CH_2); 5.00-5.13 (m, 1H, CH); 6.80 (d, 1H, *J* = 8.8 Hz, Ar); 7.24-7.40 (m, 5H, Ar); 7.60 (d, 1H, *J* = 2.4 Hz, Ar) ppm.

ethyl[2-(2,4-dichlorophenyl)-2'-oxo-2,3-dihydro-3'H-spiro[chromene-4,5'-[1,3] oxazolidin]-3'-yl]acetate 96a
Compound **96a** was obtained from **91a** (0.17 g, 0.5 mmol) and ethylbromoacetate (0.08 g, 0.5 mmol) following the procedure described for **67a**.The crude product was directly used in the next step without further purification. **96a** (0.14 g, 0.32 mmol, yield 65%). ^{1}H-NMR ($CDCl_3$) δ : 1.25-1.34 (m, 3H, Me); 2.25-2.41 (m, 1H, CH_2); 2.62-2.70 (m, 1H, CH_2); 3.83-4.01 (m, 2H, CH_2); 4.10-4.13 (m, 2H, CH_2); 4.19-4.30 (m, 2H, CH_2); 5.40-5.51 (m, 1H, CH); 6.92-6.96 (m, 1H, Ar); 7.04-7.11 (m, 1H, Ar); 7.30-7.43 (m, 2H, Ar); 7.54 (d, 1H, *J* = 7.7 Hz, Ar); 7.68 (d, 1H, *J* = 8.4 Hz, Ar) ppm.

ethyl[6-bromo-2-(2,4-dichlorophenyl)-2'-oxo-2,3-dihydro-3'H-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetate 96b.
Compound **96b** was obtained from **91b** (0.4 g, 0.93 mmol) and ethylbromoacetate (0.15 g, 0.93 mmol) following the procedure described for **67a**.The crude product was directly used in the next step without further purification. **96b** (0.38 g, 0.73 mmol, yield 79 %): ^{1}H-NMR ($CDCl_3$) δ : 1.21-1.34 (m, 3H, Me); 2.18-2.33 (m, 1H, CH_2); 2.57-2.68 (m, 1H, CH_2); 3.82-3.91 (m, 2H, CH_2); 4.06-4.20 (m, 2H, CH_2); 4.20-4.30 (m, 2H, CH_2); 5.40 (d, 1H, *J* = 12.3 Hz, CH); 6.83 (d, 1H, *J* = 8.8 Hz, Ar); 7.34-7.43 (m, 3H, Ar); 7.58-7.65 (m, 2H, Ar) ppm.

ethyl[6-bromo-2-(2,4-difluorophenyl)-2'-oxo-2,3-dihydro-3'H-spiro[chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetate 97b.
Compound **97b** was obtained from **92b** (0.3 g, 0.77 mmol) and ethylbromoacetate (0.13 g, 0.77mmol) following the procedure described for

67a.The crude product was purified by flash column chromatography eluted with Hex/EtOAc (7/3) to give **97b** (0.09 g, 0.18 mmol, yield 24%): ^{1}H-NMR ($CDCl_3$) δ : 1.31 (t, 3H, *J* = 7.1 Hz, Me); 2.41-2.59 (m, 2H, CH_2); 3.84 (d, 1H, *J* = 8.4 Hz, CH_2); 3.92 (d, 1H, *J* = 8.4 Hz, CH_2); 4.14 (d, 2H, *J* = 3.0 Hz, CH_2); 4.26 (q, 2H, *J* = 7.1 Hz, CH_2); 5.31-5.38 (m, 1H, CH); 6.82 (d, 1H, *J* = 8.8 Hz, Ar); 6.87-7.11 (m, 2H, Ar); 7.38 (dd, 1H, *J* = 2.3, 8.8 Hz, Ar); 7.51-7.60 (m, 1H, Ar); 7.62 (d, 1H, *J* = 2.3 Hz, Ar) ppm.

ethyl[6-bromo-2-(4-bromo-2-fluorophenyl)-2'-oxo-2,3-dihydro-3'H-spiro [chromene-4,5'-[1,3]oxazolidin]-3'-yl]acetate 98b.
Compound **98b** was obtained from **93b** (0.35 g, 0.77 mmol) and ethylbromoacetate (0.13 g, 0.77 mmol) following the procedure described for **67a**.The crude product was directly used in the next step without further purification. **93b** (0.38 g, 0.7 mmol, yield 87%): ^{1}H-NMR ($CDCl_3$) δ : 1.32 (t, 3H, *J* = 7.1 Hz, Me); 2.38-2.54 (m, 2H, CH_2); 3.81-3.95 (m, 2H, CH_2); 4.12 (s, 2H, CH_2); 4.26 (q, 2H, *J* = 7.1 Hz,; CH_2); 5.32-5.45 (m, 1H, CH); 6.83 (d, 1H, *J* = 8.8 Hz, Ar); 7.07-7.16 (m, 1H, Ar); 7.33-7.54 (m, 3H, Ar); 7.62 (d, 1H, *J* = 2.1 Hz, Ar) ppm.

1-{2-[(4-methoxybenzyl)oxy]phenyl}ethanone 102a
To a solution of 2-hydroxyacetophenone (1.5 g, 11.03 mmol) in DMSO (7 mL) was added KOH (1.86 g, 33.09 mmol) and the resulting mixture was stirred at 50°C for 15 min. After cooling to rt 4-methoxybenzyl chloride (1.73 g, 11.03 mmol) was added. The reaction mixture was stirred at rt for 4h and then washed with H_2O and $NaHCO_{3sat}$. The organic layer was dried, filtered and evaporated under vacuum to give …a without further purification. **102a** (1.5 g, 5.83 mmol, yield 53%): ^{1}H-NMR ($CDCl_3$) δ : 2.57 (s, 3H, Me); 3.83 (s, 3H, OMe); 5.09 (s, 2H, OCH_2); 6.93 (d, 2H, *J* = 8.6 Hz, Ar); 6.96-7.05 (m, 2H, Ar); 7.36 (d, 2H, *J* = 8.6 Hz, Ar); 7.40-7.49 (m, 1H, Ar); 7.75 (dd, 1H, *J* = 2.0, 7.7, Hz, Ar) ppm.

1-{2-[(3,4-dimethoxybenzyl)oxy]phenyl}ethanone 103a
Compound **103a** was synthesised from 2-hydroxyacetophenone (1.5 g, 11.03 mmol) and 3,4-dimethoxybenzyl chloride (2.06 g, 11.03 mmol) following the same procedure described for **102a**. The crude product was directly used for the next reaction without further purification.**103a** (1.83 g, 9.81 mmol, yield 89%): ^{1}H-NMR ($CDCl_3$) δ : 2.59 (s, 3H, Me); 3.89 (s, 3H, OMe); 3.90 (s, 3H, OMe); 5.09 (s, 2H, OCH_2); 6.85-7.05 (m, 5H, Ar); 7.41-7.49 (m, 1H, Ar); 7.74 (dd; 1H, *J* =1.8, 7.5 Hz, Ar) ppm.

1-{2-[(3,4,5-trimethoxybenzyl)oxy]phenyl}ethanone 104a
Compound **104a** was synthesised from 2-hydroxyacetophenone (0.9 g, mmol) and 3,4,5-trimethoxybenzyl chloride (1.5 g, 6.93 mmol) following the same procedure described for **102a**. The crude product was directly used for the next

reaction without further purification. **104a** (1.9 g, 6.03 mmol, yield 87%): ^{1}H-NMR ($CDCl_3$) δ : 2.63 (s, 3H, Me); 3.86 (s, 9H, OMe); 5.09 (s, 2H, CH_2); 6.67 (s, 2H, Ar); 6.99-7.06 (m, 2H, Ar); 7.41-7.50 (m, 1H, Ar); 7.74 (dd; 1H, *J* = 1.7, 8.0 Hz, Ar) ppm.

2-[2-(benzyloxy)phenyl]-2- [(trimethylsilyl)oxy] propanenitrile 105a
Compound **105a** was synthesised from **102a** (1.5 g, 5.83 mmol) following the procedure described for **49a**. The crude product was directly used in the next step without further purification. **105a** (1.74 g, 4.88 mmol, yield 84%): ^{1}H-NMR ($CDCl_3$) δ : 0.31 (s, 9H, Me); 1.89 (s, 3H, Me); 3.82 (s, 3H, OMe); 5.05-5.16 (m, 2H, CH_2); 6.77-7.04 (m, 4H, Ar); 7.19-7.35 (m, 1H, Ar); 7.46 (d, 2H, *J* = 8.8 Hz; Ar); 7.56 (dd, 1H, *J* = 2.0, 8.1 Hz, Ar) ppm.

2-{2-[(3,4-dimethoxybenzyl)oxy]phenyl}-2-[(trimethylsilyl)oxy] propanenitrile 106a
Compound **106a** was synthesised from **103a** (1.09 g, 5.83 mmol) following the procedure described for **49a**. The crude product was directly used in the next step without further purification. **106a** (1.91 g, 4.95 mmol, yield 85%): ^{1}H-NMR ($CDCl_3$) δ : 0.29 (s, 9H, Me); 1.89 (s, 3H, Me); 3.89 (s, 3H, OMe); 3.90 (s, 3H, OMe); 5.03-5.18 (m, 2H, CH_2); 6.67-7.03 (m, 5H, Ar); 7.18-7.41 (m, 1H, Ar); 7.56 (dd, 1H, *J* =1.8, 8.1 Hz) ppm.

2-[(trimethylsilyl)oxy]-2-{2-[(3,4,5-trimethoxybenzyl)oxy]phenyl}propane nitrile 107a
Compound **107a** was synthesised from **104a** (1.90 g, 6.03 mmol) following the procedure described for **49a**. The crude product was directly used in the next step without further purification. **107a** (2.22 g, 5.37 mmol, yield 89%): ^{1}H-NMR ($CDCl_3$) δ : 0.06 (s, 9H, Me); 2.01 (s, 3H, Me); 3.88 (s, 9H, OMe); 5.18 (s, 2H, CH_2); 6.80 (s, 2H, Ar); 7.01-7.08 (m, 2H, Ar); 7.35-7.42 (m, 2H, Ar) ppm.

1-amino-2-{2-[(4-methoxybenzyl)oxy]phenyl}propan-2-ol 108a
Compound **108a** was synthesised from **105a** (1.74 g, 4.88 mmol) following the procedure described for **50a**. The crude product was directly used in the next step without further purification. **108a** (0.83 g, 2.88 mmol, yield 59%)^{1}H-NMR ($CDCl_3$) δ : 1.49 (s, 3H, Me); 3.31 (d, 1H, *J* = 12.7 Hz, CH_2); 3.51 (d, 1H, *J* = 12.7 Hz, CH_2); 3.83 (s, 3H, OMe); 5.00-5.14 (m, 2H, CH_2); 6.87-7.05 (m, 4H, Ar); 7.18-7.28 (m, 1H, Ar); 7.35 (d, 2H, *J* = 8.6 Hz, Ar); 7.68 (d, 1H, *J* = 8.1, 2.0, Ar) ppm.
1-amino-2-{2-[(3,4-dimethoxybenzyl)oxy]phenyl}propan-2-ol 109a
Compound **109a** was synthesised from **106a** (1.91 g, 4.95 mmol) following the procedure described for **50a**. The crude product was directly used in the next step without further purification. **109a** (1.19 g, 3.76 mmol, yield 76%): ^{1}H-

NMR ($CDCl_3$) δ : 1.54 (s, 3H, Me); 2.77 (d, 1H, *J* =12.7 Hz, CH_2); 3.29 (d, 1H, *J*=12.7 Hz, CH_2); 3,88 (s, 3H, OMe); 3,89 (s, 3H, OMe); 5.03 (s, 2H, CH_2); 6.62-7.02 (m, 5H, Ar); 7.20-7.28 (m, 1H, Ar); 7.45-7.50 (m, 1H, Ar) ppm.

1-amino-2-{2-[(3,4,5-trimethoxybenzyl)oxy]phenyl}propan-2-ol 110a
Compound **110a** was synthesised from **107a** (2.22 g, 5.37 mmol) following the procedure described for **50a**. The crude product was directly used in the next step without further purification. **110a** (1.21 g, 3.49 mmol, yield 65%) ^{1}H-NMR ($CDCl_3$) δ : 1.57 (s, 3H, Me); 2.80 (d, 1H, *J* =12.7 Hz, CH_2); 3.31 (d, 1H, *J*=12.7 Hz, CH_2); 3.85 (s, 9H, OMe); 5.03 (s, 2H, CH_2); 6.65 (s, 2H, Ar); 6.90-7.02 (m, 2H, Ar); 7.18-7.28 (m, 1H, Ar); 7.48-7.53 (m, 1H, Ar) ppm.

5-{2-[(4-methoxybenzyl)oxy]phenyl}-5-methyl-1,3-oxazolidin-2-one 111a
Compound **111a** was synthesised from **108a** (0.48 g, 1.67 mmol) following the procedure described for **57a**. The crude product was purified by flash column chromatography eluted by Hex/EtOAc (5.5/4.5) to give **111a** (0.16 g, 0.5 mmol, yield 30%): ^{1}H-NMR ($CDCl_3$) δ : 1.73 (s, 3H, Me); 3.62 (d, 1H, *J* = 9.1 Hz, CH_2); 3.72 (d, 1H, *J* = 9.1 Hz, CH_2); 3.83 (s, 3H, OMe); 4.99 (s, 2H, OCH_2); 6.93 (d, 2H, *J* = 8.4 Hz, Ar); 6.96-7.04 (m, 2H, Ar); 7.25-7.33 (m, 3H, Ar); 7.59-7.63 (m, 1H, Ar) ppm.

5-{2-[(3,4-dimethoxybenzyl)oxy]phenyl}-5-methyl-1,3-oxazolidin-2-one 112a
Compound **112a** was synthesised from **109a** (0.53 g, 1.67 mmol) following the procedure described for **57a**. The crude product was directly used in the next step without further purification. **112a** (0.45 g, 1.30 mmol, yield 78%): ^{1}H-NMR ($CDCl_3$) δ: 1.75 (s, 3H, Me); 3.63 (d, 1H, *J* = 9,1 Hz, CH_2); 3.73 (d, 1H, *J* = 9,1 Hz, CH_2); 3.89 (s, 3H, OMe); 3.90 (s, 3H, OMe); 4.92-5.09 (m, 2H, CH_2); 6.70-7.04 (m, 5H, Ar); 7.25-7.33 (m, 1H, Ar); 7.58-7.63 (m, 1H, Ar) ppm.

5-methyl-5-{2-[(3,4,5-trimethoxybenzyl)oxy]phenyl}-1,3-oxazolidin-2-one 113a
Compound **113a** was synthesised from **110** (1.21 g, 3.49 mmol) following the procedure described for **57a**. The crude product was directly used in the next step without further purification. **113a** (1.07 g, 2.86 mmol, yield 82%): ^{1}H-NMR ($CDCl_3$) δ: 1.80 (s, 3H, Me); 3.67-3.93 (m, 11H, CH_2, OMe); 4.92-5.45 (m, 2H, CH_2); 6.60 (s, 2H, Ar); 6.92-7.06 (m, 2H, Ar); 7.20-7.30 (m, 1H, Ar); 7.62 (dd, 1H, *J* = 1.6, 7.7 Hz, Ar) ppm.

ethyl(5-{2-[(4-methoxybenzyl)oxy]phenyl}-5-methyl-2-oxo-1,3-oxazolidin-3-yl) acetate 114a

Compound **114a** was synthesised from **111a** (0.16 g, 0.5 mmol) and ethylbromoacetate (0.08 g, 0.5 mmol) following the procedure described for **67a**. The crude product was directly used in the next step without further purification. **114a** (0.16 g, 0.4 mmol, yield 81%): ^{1}H-NMR ($CDCl_3$) δ:1.18-1.27 (m, 3H, Me); 1.77 (s, 3H, Me); 3.66-3.78 (m, 2H, CH_2); 3.83 (s, 3H, OMe); 4.02-4.26 (m, 4H, CH_2); 5.01 (s, 2H, OCH_2); 6.91-7.04 (m, 4H, Ar); 7.26-7.33 (m, 3H, Ar); 7.59-7.63 (m, 1H, Ar) ppm.

ethyl(5-{2-[(3,4-dimethoxybenzyl)oxy]phenyl}-5-methyl-2-oxo-1,3-oxazolidin-3-yl)acetate 115a

Compound **115a** was synthesised from **112a** (0.17 g, 0.5 mmol) and ethylbromoacetate (0.08 g, 0.5 mmol) following the procedure described for **67a**. The crude product was directly used in the next step without further purification. **115a** (0.17 g, 0.4 mmol, yield 81%): ^{1}H-NMR ($CDCl_3$) δ:1.14-1.33 (m, 3H, Me); 1.79 (s, 3H, Me); 3.65-3.98 (m, 8H, CH_2, OMe); 4.02-4.25 (m, 2H, CH_2); 5.01 (s, 2H, OCH_2); 6.70-6.79 (m, 1H, Ar); 6.83-7.03 (m, 4H, Ar); 7.24-7.33 (m, 1H, Ar); 7.58-7.62 (m, 1H, Ar) ppm.

ethyl(5-{2-[(3,4,5-trimethoxybenzyl)oxy]phenyl}-5-methyl-2-oxo-1,3-oxazolidin -3-yl) acetate 116a

Compound **116a** was synthesised from **113a** (0.50 g, 1.34 mmol) and ethylbromoacetate (0.22 g, 1.34 mmol) following the procedure described for **67a**. The crude product was directly used in the next step without further purification. **116a** (0.54 g, 1.18 mmol, yield 88%): ^{1}H-NMR ($CDCl_3$) δ:1.14-1.33 (m, 3H, Me); 1.84 (s, 3H, CH_3); 3.70-3.92 (m, 11H, CH_2, OMe); 4.06-4.22 (m, 2H, CH_2); 4.94-5.17 (m, 2H, CH_2); 6.61 (s, 2H, Ar); 6.93-7.05 (m, 2H, Ar); 7.20-7.30 (m, 1H, Ar); 7.63 (dd, 1H, J = 1.6, 7.7 Hz, Ar) ppm.

3,4-dimethoxybenzyl chloride 100

To a stirred solution of 3,4-dimethoxybenzyl alcohol (1.72 g, 10.26 mmol) in $CHCl_3$ (20 mL) was added dropwise $SOCl_2$ (2.24 ml, 30.78 mmol) at 0 ºC. The resulting mixture was stirred rt for 20h, then washed with H_2O and brine. The organic phase was dried, filtered and the solvent was evaporated under vacuum to give **100** (1.65 g, 8.83 mmol, yield 86 %). ^{1}H-NMR ($CDCl_3$) δ: 3.85 (s, 3H, OCH_3); 3.87 (s, 3H, OCH_3); 4.54 (s, 2H, CH_2); 6.78-6.94 (m, 3H, Ar) ppm.

3,4,5-trimethoxybenzyl chloride 101

Compound **101** was synthesised from 3,4-dimethoxybenzyl alcohol (2.00 g, 10.10 mmol) following the same procedure described for **100**. The crude product was used in the next reaction without further purification. **101** (1.94 g, 8.99 mmol, yield 89%).^{1}H-NMR ($CDCl_3$) δ: 3.85 (s, 3H, OCH_3); 3.87 (s, 3H, OCH_3); 4.54 (s, 2H, CH_2); 6.78-6.94 (m, 3H, Ar) ppm.

5.2 Enantiomeric resolution

HPLC enantioseparations were performed by using stainless-steel Chiralpak IA (250 x 4.6 mm I.D. and 250 x 10 mm I.D.) (Daicel, Chemical Industries, Tokyo, *J*apan) columns. HPLC-grade solvents were supplied by Carlo Erba (Milan, Italy). HPLC apparatus consisted in a Perkin Elmer (Norwalk, CT, USA) 200 lc pump equipped with a Rheodyne (Cotati, CA, USA) in*J*ector, a HPLC Dionex (CA, USA) Model TCC-100 oven and a *J*asco (*J*asco, Ishikawa-cho, Hachio*J*i City, Tokyo, *J*apan) Model 2095 Plus UV/CD detector.

The HPLC enantioseparation of compounds **1a** and **2b** was carried out on the amylose-based Chiralpak IA 250 x 4.6 mm I.D (for evaluation of chromatographic data and enantiomeric excess) and 250 x 10 mm I.D. (for semipreparative enantioseparations); temperature, 25°C, flow-rate, 1 ml/min (for analytical enantioseparations) and 5 ml/min (for semipreparative enantioseparations); detection: UV at 225 nm (for analytical enantioseparation of **1a**); 280 nm (for analytical enantioseparation of **2b**); 290 and 300 nm (for semipreparative enantioseparations of **2b** and **1a**, respectively).

The mobile phases were filtered and degassed by sonication immediately before using. In analytical enantioseparations, standard solutions were prepared by dissolving about 2 mg of sample, into 10 ml of mobile phase. The in*J*ection volume was 10-20 μl. In semipreparative enantioseparation a 1 mL sample loop was used. After semipreparative separation, the collected fractions were analyzed by chiral analytical columns to determine their enantiomeric excess (ee).

The column hold-up time (t_0 = 3.0 min for 250 x 4.6 mm I.D column) was determined from the elution of an unretained marker (toluene); using ethanol as eluent, delivered at a flow-rate of 1.0 mL/min.

The eluent composition and the corresponding analytical chromatographic data for each resolved compound are summarized as follows: **1a**: n-Hexane-ethanol 75/25 (v/v); k_1 = 2.27, α = 1.45, Rs = 4.55; **2b**: n-Hexane-ethyl acetate-trifluoroacetic acid 40/60/0.1 (v/v/v); k_1 = 0.88, α = 1.36, Rs = 2.92. k_1: retention factor of the first eluted enantiomer, defined as $(t_1 - t_0)/t_0$ where t_0 is the void time of the column; α: enantioselectivity factor defined as k_2/k_1; Rs: resolution factor defined as $2(t_2-t_1)/(w_1+w_2)$ where t_1 and t_2 are retention times and w_1 and w_2 are band widths at the baseline in time units. Other analytical chromatographic conditions: flow-rate, 1.0 mL/min; temperature, 25 °C; detector: UV and CD at 225 nm (for **1a**) and 280 nm (for **2b**).

Specific rotations of enantiomers of **1a** and **2b**, dissolved in ethanol, were measured at 589 nm by a Perkin-Elmer polarimeter model 241 equipped with a Na lamp. The volume of the cell was 1 ml and the optical path was 10 cm. The system was at a temperature of 20°C by a Neslab RTE 740 cryostat. The circular dichroism (CD) spectra of stereoisomers of **1a** and **2b**, dissolved in ethanol (concentration about 0.2 mg/mL); in a quartz cell (0.1 cm-path length)

at 25°C, were measured by using a *J*asco Model *J*-700 spectropolarimeter. The spectra are average computed over three instrumental scans and the intensities are presented in terms of ellipticity values (mdeg).
RX-analysis was carried out with a Goniometer Oxford Diffraction KM4 Xcalibur2 at room temperature.
Graphite-monochromated Mo/Kα radiation (40mA/-40KV) and a KM4 CCD/SAPPHIRE detector were used for cell parameter determination and data collection.
The integrated intensities, measured using the ω scan mode, were corrected for Lorentz and polarization effects.[10] The substantial redundancy in data allows empirical absorption corrections (SADABS)[11] to be applied using multiple measurements of symmetry-equivalent reflections.
The structure was solved by direct methods of SIR2004[12] and refined using the full-matrix least squares on F^2 provided by SHELXL97.[13]
The stereochemical correspondences between the couples of the isolated enantiomers of **1a** and **2b** were established by CD analysis. The structures and the absolute configurations of (+)-**2b** and (-)-**2b,** and therefore of (+)-**1a** and (-)-**1a**, was readily secured by X-ray crystallography and dextrorotatory and levorotatory enantiomers showed to have (*R*) and (*S*) configuration, respectively.

X-ray structural analysis of compounds R-(+)-2b and S-(-)-2b
R-(+)-2b. $C_{22}H_{25}N_2O_5BrS$, M =509.41, Trigonal, space group P31, *a* =12.161(1); *b* =12.161(1); *c* =13.193(1)Å, V =1689.7(2)$Å^3$,Z =3 D_c =1.502, μ =1.955 mm^{-1}, F(000) =786.
8157 reflections were collected with a 4.40<θ< 31.92 range; 6123 were independent; the parameters were 298 and the final R index was 0.0571 for reflections having I>2σI and 0.0967 for all data.
S-(-)-2b. $C_{22}H_{25}N_2O_5BrS$, M =509.41, Trigonal, space group P31, *a* =12.161(1); *b* =12.161(1); *c* =13.184(1)Å, V =1688.6(2)$Å^3$, Z =3 D_c =1.503, μ =1.953 mm^{-1}, F(000) =786.
3906 reflections were collected with a 4.56<θ< 28.75 range; 2862 were independent; the parameters were 284 and the final R index was 0.0464 for reflections having I>2σI and 0.0959 for all data.
In both compounds the non-hydrogen atoms were refined anisotropically. Aromatic, methylic and methylenic hydrogens were assigned in calculated positions, whereas hydrogen on N_2 was found in the Fourier synthesis; all of them were refined as isotropic.

5.3 Pharmacological procedures

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609.

In vitro vascular protocols

The effects of the compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250-350 g).

After a light ether anaesthesia, the rats were sacrificed by cervical dislocation and bleeding.

The aortas were immediately excised and freed of extraneous tissues, and the endothelial layer was removed by gently rubbing the intimal surface of the vessels with a hypodermic needle. Five millimeter wide aortic rings were suspended, under a preload of 2 g, in 20 mL organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4$ $7H_2O$ 1.05; NaH_2PO_4 0.41; $NaHCO_3$ 11.9; Glucose 5.5); thermostated at 37°C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Grass FTO3); connected with a preamplifier (Buxco Electronics) and with a software of data acquisition (BIOPAC Systems Inc., MP 100).

After an equilibration period of 60 minutes, endothelium removal was confirmed by the administration of acetylcholine (ACh) (10 μM) to KCl (20 mM)-precontracted rings. A relaxation < 10% of the KCl-induced contraction was considered to be indicative of an acceptable lack of the endothelial layer, while the organs showing a relaxation ≥ 10% (i.e., significant presence of the endothelium) were discarded.

From 30 to 40 minutes after the confirmation of the endothelium removal, the aortic preparations were contracted by a single concentration of KCl (20mM); and when the contraction reached a stable *plateau*, 3-fold increasing concentrations of the test substances (from 10 nM to 100 μM) were added.

Preliminary experiments showed that the KCl (20 mM)-induced contractions remained in a stable tonic state for at least 40 minutes.

Data analysis

The vasorelaxing efficacy was evaluated as the maximal vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by 20mM KCl. When the limit concentration of 100 μM (the highest concentration that could be administered) of the tested compounds did not reach the maximal effect, the parameter of efficacy represented the vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by 20mM KCl, evoked by this limit concentration. The parameter of potency was expressed as pIC_{50}, calculated as negative logarithm of the molar concentration of the test

compounds, evoking a 50% reduction of the contractile tone induced by 20mM KCl. The pIC_{50} could not be calculated for those compounds showing an efficacy parameter lower than 50%. The parameters of efficacy and potency were expressed as means ± standard error, for 5-10 experiments. Student *t* test was selected for statistical analysis, $P < 0.05$ was considered to be indicative of a significant statistical differences. Experimental data were analysed by a computer fitting procedure (software: GraphPad Prism 4.0).

In vitro cardiac protocols

Adult male Wistar rats (260-350 g) were treated by an i.p. in*J*ection (about 0.3 ml) with a dose of 40mg/Kg with the following compounds: diazoxide, **1a,b**, **2b**, **3a,b**, **4b**, vehicle (DMSO) or with a dose of 1mg/Kg as concerns cromakalim. After 2 hours, all the animals were anaesthetised with sodium pentobarbital (100 mg/Kg i.p.) and heparinised (100 UI i.p.) to prevent blood clotting. In order to verify the effective selectivity towards the mitochondrial K_{ATP} channels, in another series of experiments a selective mito-K_{ATP} blocker was administered, in a 10mg/Kg dose to the rats, 20 minutes before the administration of the tested compounds. After the opening of the chest, the hearts were quickly excised and placed in a 4°C Krebs solution (composition mM: $NaHCO_3$ 25.0, NaCl 118.1, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2.2H_2O$ 1.6, KH_2PO_4 1.2, glucose 11.5) equilibrated with 95% O_2 5% CO_2, to stop the contraction and to reduce oxygen consumption. Rapidly, the ascending aorta was cannulated and hearts mounted on a Langendorff apparatus, then the perfusion with Krebs solution (thermostated at 37°C and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2) was started at constant pressure (70-80 mmHg). The above procedure was executed within 2 min. A water-filled latex balloon connected to a pressure transducer (Bentley Trantec, mod 800) was introduced into the left ventricle via the mitral valve and the volume was ad*J*usted to achieve a stable left ventricular end-diastolic pressure of 5-10 mmHg during initial equilibration. The heart rate (HR) and left ventricular developed pressure (LVDP) were monitored by a Biopac system (California, USA) and the parameter of Rate Pressure Product (RPP) was calculated as RPP = HR x LVDP. In order to avoid that a physiological fall in the contractile activity, due to a long period of reperfusion, (which could cause a damage not exclusively related to ischemia) could influence the corrected analysis of the functional data, the RPP value recorded at the 30^{th} minute of reperfusion (RPP-30') was considered more reliable than the RPP value recorded at the last minute of reperfusion (RPP-120'). The RPP-30' and the RPP-120' parameters have been expressed as % of the RPP value recorded at the last minute of the pre-ischemic period. The coronary flow (CF) was measured volumetrically and was expressed as mL of the perfusate collected in 1 min. After a 30 min equilibration pre-ischemic period, the hearts were sub*J*ected to 30 min of global ischemia (no flow). Hearts showing severe arrhythmia or unstable LVDP and

HR values were excluded from the experiments. At the end of the ischemic period the hearts were reperfused for a period of 120 minutes. Moreover, the lactate de-hydrogenase enzyme (LDH, a biochemical marker of the ischemic damage) collected in the last 5 minutes of the pre-ischemic phase and that collected every five minutes during all the reperfusion period, was measured by a spectrophotometric method. The amount of released LDH has been expressed in enzymatic mU released in 120 minutes of reperfusion (without the small amount recorded in the pre-ischemic phase); resulting from the AUC analysis (area under curve in the Cartesian graph of the LDH amount recorded at the determined intervals *vs* time) and related to1 g of the heart weight. At the end of the reperfusion period the hearts were removed from the Langendorff apparatus and the left ventricle has been cut in 2mm large slices which first have been bathed in a 10% aqueous solution of 2,3,5-triphenyltetrazolium chloride (TTC) for 20 minutes and then in a 20% aqueous solution of formaldehyde. After 24 hours, the ventricular slices have been photographed and analysed in order to highlight the necrotic areas due to the ischemic process (visible as white or light pink colour) and the healthy areas (visible as strong red due to the TCC reaction) and then to calculate the % of ischemic area in front of the total myocardial area.

In vivo model of coronary occlusion-reperfusion

Two hours before the experimental procedures, rats received an i.p. in*J*ection of compound **a** (2, 10 or 40 mg/Kg); reference drug diazoxide (40 mg/Kg, Sigma-Aldrich); or vehicle (dimethylsulphoxide, DMSO, Sigma-Aldrich). When required by the experimental protocol, the mito-K_{ATP} blocker, 5-HD (10 mg/Kg, Sigma-Aldrich); was in*J*ected 20 min before the treatment with the mito-K_{ATP} openers.

Then, rats were anesthetised with sodium pentobarbital (70 mg/kg, i.p.). The trachea was intubated and connected to a rodent ventilator (mod. 7025 Ugo Basile, Comerio-Italy) for artificial ventilation with room air (stroke volume, 1 ml/100 g of animal body weight; 70 strokes/min). Electrocardiogram (ECG) was continuously measured by lead II (Mindray, PM5000). The chest was opened by a left thoracotomy and the heart was gently exteriorized. A 6-0 surgical needle was passed around the left anterior descending coronary artery (LAD); located between the base of the pulmonary artery and left atrium. The ends of the suture were passed through a polypropylene tube (PE50) to form a snare. To induce infarction, the LAD was occluded by pulling the snare and then fixing it in place by clamping the tube with a haemostat, when the snare was released the reperfusion was initiated. The acute infarct protocol consisted of 30’ occlusion/120’ reperfusion, successful occlusion was confirmed visually by regional cyanosis downstream of the ligature and by ST elevation on the ECG.

A group of animals was submitted to an IPC procedure, achieved by 2 cycles of 5' occlusion/10' reperfusion, followed by 30' coronary occlusion and 120' reperfusion. Each experimental group was composed by at least 6 animals. At the end of reperfusion, rats were euthanised by an overdose of pentobarbital sodium, then hearts were quickly excised, mounted on a Langendorff apparatus and perfused for 10' with Krebs solution to wash out the coronary blood vessels. Then, hearts were deprived of atria and right ventricle.
The left ventricular tissue was dried, frozen, and cut into 4-5 transverse slices from apex to base of equal thickness (about 2 mm). The slices were then incubated in a 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) solution in a phosphate buffer (pH 7.4) at 37 °C for 20 min.
TTC reacts with NADH in the presence of dehydrogenase enzymes, to form a formazan derivative, thus, causing the staining of viable cells with intense red colour.
Subsequently, the slices were fixed overnight in 10% formaldehyde and then were photographed. Red-stained viable tissue was easily distinguished from the infracted white-unstained necrotic tissue. The infarct area (Ai) was planimetrically evaluated using an image analyzer program (The GIMP 2). The infarct size was calculated as a percentage of the whole area of left ventricle ($Ai/A_{LV}\%$).

Cell culture
H9c2 cells, derived from embryonic rat ventricular myocytes (ATTC, Manassas, VA); were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich); 100 units/ml penicillin and 100µg/ml streptomycin in tissue culture flasks at 37°C in humidified atmosphere of 5% CO_2.
H9c2 cells were cultured up to about 80% confluence in DMEM medium and 24 hours before the experiment, cells were seeded onto 96-well plates at a density of 8×10^3 per well. After 24 hours to allow cell attachment, the medium was replaced in each 96-well plate and cells received different treatments such as: vehicle (that is 0.05% DMSO); diazoxide 100µM, compound a 10µM, 5-HD 500 µM plus diazoxide 100 µM, 5-HD plus compound a 10µM. Then, one plate was placed for 16 hours in an airtight AtmosBag (Sigma-Aldrich) which was saturated with 95% N_2 and 5% CO_2 to simulate ischemia, while the twin plate was placed for 16 hours in an airtight AtmosBag which was saturated with 95% air and 5% CO_2. At the end of the hypoxia period all the cells were sub*J*ected to reperfusion by replacing of the medium with the culture DMEM above described and incubation for 2 hours in an athmosphere containing 5% CO_2 / 95 % air at 37°C. After reperfusion, cell viability was assessed using the Cell Proliferation Reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolium]-1,3-benzene disulphonate) (Roche, Mannheim, Germany) based on the cellular cleavage of the WST-1 to formazan. WST-1 Reagent was added at

1/10 of the total volume and after 60 min of incubation at 37°C, the absorbance was measured at 450 nm with a microplate reader (Wallac; PerkinElmer, Wellesley, MA, USA). Absorbance values have been normalised as a % of the absorbance shown by vehicle-treated cells exposed in the non-hypoxic atmosphere (Viab. %). Three different experiments have been performed in sixtuplicate.

Isolation of rat mitochondria

Rat cardiac mitochondria were isolated by differential centrifugation in agreement with the method of Chappel and Hansford [21]. Male Wistar rats were killed by cervical dislocation, the heart was removed immediately and placed in ice cold isolation buffer (composition: sucrose 250 mM, Tris 5 mM, EGTA 1mM, pH 7.4 adJusted with HCl). Atria were removed and ventricular tissue was finely minced with surgical scissors (about 2 mm^3 pieces) and homogenised using an Ultra-Turrax homogeniser (20 ml of isolation buffer per heart).

Three 20s homogenisation cycles were performed on ice, and then the suspension was centrifuged at 1075xg for 3 min at 4°C. The resulting supernatant was centrifuged at 11950xg for 10 min at 4°C. The pellet containing the mitochondrial fraction was further re-suspended in isolation buffer (without EGTA) and centrifuged at 11950xg for 10 min at 4°C, this step was repeated once more.

The final mitochondrial pellet was re-suspended in minimal volume of 400 µl of the isolation buffer (without EGTA) and stored on ice throughout the experiment, which was performed within 2 hours. Mitochondrial protein concentrations were determined using the Bradford reaction [22].

Previous experiments (data not shown) confirmed the reliability of the isolation procedures, by measurement of mitochondrial respiratory function through ATP bioluminescence assay, in agreement with the method of Drew and Leeuwenburgh [23]. Briefly, mitochondria (1 mg protein/ml) were suspended in isolation buffer (without EGTA) plus succinate 20 mM, KH_2PO_4 30 mM and ADP 5' diphosphate 200 µM. The reaction was initiated by the addition of luciferin-luciferase reagent. ADP 5' diphosphate replaced by bidistilled water was used as a blank. 3 min after the start of reaction, the measurement was performed using a luminometer (Wallac, Perkin Elmer, Wellesley, MA, USA). In all the tested preparations, the assay really detected the light produced by the ATP-dependent oxidation of luciferin by means of luciferase enzyme. The ATP-synthase inhibitor olygomicin 2 µg/ml (Sigma-Aldrich) or the uncoupler of oxidative phosphorylation 2,4-dinitrophenol (DNF, 100 µM, Sigma-Aldrich) inhibited ATP production.

Mitochondrial swelling
Mitochondrial swelling was assessed spectrophotometrically by measuring the change in absorbance of the suspension at 520 nm (Wallac, Perkin Elmer, Wellesley, MA, USA).
Mitochondria (1mg protein/ml) were suspended under gently stirring in incubation medium (composition: KCl 120 mM, K_2HPO_4 5 mM, Hepes 10 mM, succinic acid 10 mM, $MgCl_2$ 2 mM, ATP 200 μM, pH 7.4 adJusted with KOH). The decrease of absorbance was monitored for 15 min after the addition of a (100μM); diazoxide (100 μM); or vehicle (DMSO 1%). Each time-response curve was obtained with mitochondria isolated from hearts of at least 6 different animals.

Enzymatic inhibition
Aldose reductase (ALR2) and aldehyde reductase (ALR1) were obtained from Sprague-Dawley albino rats, 120-140 g body weight, supplied by Harlan Nossan, Italy. To minimize cross-contamination between ALR2 and ALR1 in the enzyme preparation, rat lens, in which ALR2 is the predominant enzyme, and kidney, where ALR1 shows the highest concentration, were used for the isolation of ALR2 and ALR1, respectively. Pyridine coenzyme, D,L-glyceraldehyde, and sodium D-glucuronate were from Sigma-Aldrich. Tolrestat was obtained from Lorestat Recordati, Italy. All other chemicals were of reagent grade.

Enzyme Preparation.
Aldose Reductase (ALR2). A purified rat lens extract was prepared in accordance with the method of Hayman and Kinoshita44 with slight modifications. Lenses were quickly removed from rats following euthanasia and were homogenized (Glas-Potter) in 3 volumes of cold deionized water. The homogenate was centrifuged at 12000 rpm at 0-4 °C for 30 min. Saturated ammonium sulfate solution was added to the supernatant fraction to form a 40% solution, which was stirred for 30 min at 0-4 °C and then centrifuged at 12000 rpm for 15 min. Following this same procedure, the recovered supernatant was subsequently fractionated with saturated ammonium sulfate solution using first a 50% and then a 75% salt saturation. The precipitate recovered from the 75% saturated fraction, possessing ALR2 activity, was redissolved in 0.05 M NaCl and dialyzed overnight in 0.05 M NaCl. The dialyzed material was used for the enzymatic assay.
Aldehyde Reductase (ALR1). Rat kidney ALR1 was prepared in accordance with a previously reported method.33 Kidneys were quickly removed from normal killed rats and homogenized (Glas- Potter) in 3 volumes of 10 mM sodium phosphate buffer, pH 7.2, containing 0.25 M sucrose, 2.0 mM EDTA dipotassium salt, and 2.5 mM _-mercaptoethanol. The homogenate was centrifuged at 12000 rpm at 0-4 °C for 30 min, and the supernatant was

sub*J*ected to a 40-75% ammonium sulfate fractionation, following the same procedure previously described for ALR2. The precipitate obtained from the 75% ammonium sulfate saturation, possessing ALR1
activity, was redissolved in 50 volumes of 10 mM sodium phosphate buffer, pH 7.2, containing 2.0 mM EDTA dipotassium salt and 2.0 mM _-mercaptoethanol, and was dialyzed overnight using the same buffer. The dialyzed material was used in the enzymatic assay.

Enzymatic Assays.
The activity of the two test enzymes was determined spectrophotometrically by monitoring the change in absorbance at 340 nm, which is due to the oxidation of NADPH catalyzed by ALR2 and ALR1. The change in pyridine coenzyme concentration/min was determined using a Beckman DU-64 kinetics software program (Solf Pack TM Module). ALR2 activity was assayed at 30 °C in a reaction mixture containing 0.25 mL of 10 mM d,L-glyceraldehyde, 0.25 mL of 0.104 mM NADPH, 0.25 mL of 0.1 M sodium phosphate buffer (pH 6.2); 0.1 mL of enzyme extract, and 0.15 mL of deionized water in a total volume of 1 mL. All the above reagents, except D,Lglyceraldehyde, were incubated at 30 °C for 10 min; the substrate was then added to start the reaction, which was monitored for 5 min. Enzyme activity was calibrated by diluting the enzymatic solution to obtain an average reaction rate of 0.011 (0.0010 absorbance units/min for the sample.
ALR1 activity was determined at 37 °C in a reaction mixture containing 0.25 mL of 20 mM sodium D-glucuronate, 0.25 mL of 0.12 mM NADPH, 0.25 mL of dialyzed enzymatic solution, and 0.25 mL of 0.1 M sodium phosphate buffer (pH 7.2) in a total volume of 1 mL. The enzyme activity was calibrated by diluting the dialyzed enzymatic solution to obtain an average reaction rate of 0.015 (0.0010 absorbance/min for the sample.

Enzymatic Inhibition.
The inhibitory activity of the new synthesised compounds against ALR2 and ALR1 was assayed by adding 0.1 mL of the inhibitor solution to the reaction mixture described above. All the inhibitors were solubilized in water and the solubility was facilitated by ad*J*ustment to a favorable pH. After complete solution, the pH was read*J*usted to 7. To correct for the non enzymatic oxidation of NADPH and for absorption by the compounds tested, a reference blank containing all the above assay components except the substrate was prepared. The inhibitory effect of the new derivatives was routinely estimated at a concentration of 10^{-4} M. Those compounds found to be active were tested at additional concentrations between 10^{-5} and 10^{-8} M. The determination of the IC_{50} values was performed by linear regression analysis of the log-dose response curve, which was generated using at least four concentrations of the inhibitor causing an inhibition between 20% and 80%, with two replicates at

each concentration. The 95% confidence limits (95% CL) were calculated from *t* values for *n*-2, where *n* is the total number of determinations.

5.4 Elemental composition

Compound	Formula	Calculated %			Found %		
		C	H	N	C	H	N
15a	$C_{22}H_{25}NO_4$	71.91	6.86	3.81	71.63	6.83	3.80
15b	$C_{22}H_{24}BrNO_4$	59.20	5.42	3.14	58.96	5.40	3.12
16a	$C_{22}H_{25}NO_4$	71.91	6.86	3.81	71.76	6.84	3.80
16b	$C_{22}H_{24}BrNO_4$	59.20	5.42	3.14	59.02	5.40	3.13
17a	$C_{21}H_{22}BrNO_3$	60.59	5.33	3.36	60.53	5.32	3.35
17b	$C_{21}H_{21}Br_2NO_3$	50.93	4.27	2.83	50.80	4.26	2.82
18a	$C_{22}H_{27}NO_3 \cdot HCl$	67.77	7.24	3.59	67.97	7.25	3.60
18b	$C_{22}H_{26}BrNO_3 \cdot HCl$	56.36	5.80	2.99	56.40	5.82	3.02
19a	$C_{22}H_{27}NO_3 \cdot HCl$	67.77	7.24	3.59	67.85	7.25	3.60
19b	$C_{22}H_{26}BrNO_3 \cdot HCl$	56.36	5.80	2.99	56.41	5.85	3.02
20a	$C_{21}H_{24}BrNO_2 \cdot HCl$	57.48	5.74	3.19	57.65	5.76	3.20
20b	$C_{21}H_{23}Br_2NO_2 \cdot HCl$	48.72	4.67	2.71	48.74	4.48	2.75
21a	$C_{21}H_{23}NO_2S$	71.36	6.56	3.96	71.07	6.53	3.94
21b	$C_{21}H_{22}BrNO_2S$	58.33	5.13	3.24	58.33	5.25	3.27
22a	$C_{22}H_{25}NO_2S$	71.90	6.86	3.81	71.68	6.84	3.79
23a	$C_{21}H_{22}BrNO_2S$	58.33	5.13	3.24	58.35	5.11	3.23
24a	$C_{21}H_{21}NO_4$	71.78	6.02	3.99	71.16	5.67	1.71
24b	$C_{21}H_{20}BrNO_4$	58.62	4.68	3.26	58.53	4.43	3.59
25a	$C_{20}H_{21}NO_5S$	62.00	5.46	3.62	61.05	5.32	2.25
25b	$C_{20}H_{20}BrNO_5S$	51.51	4.32	3.00	51.53	4.32	3.00

26a	$C_{21}H_{23}NO_3$	74.75	6.87	4.15	74.45	6.84	4.13
26b	$C_{21}H_{22}BrNO_3$	60.59	5.33	3.36	60.65	5.45	3.40
27a	$C_{22}H_{25}NO_3$	75.19	7.17	3.99	75.11	7.16	3.98
28a	$C_{21}H_{22}FNO_3$	70.97	6.24	3.94	71.18	6.26	3.97
29a	$C_{21}H_{23}NO_2S$	71.36	6.56	3.96	71.64	6.58	3.97
30a	$C_{21}H_{24}N_2O_3$	71.57	6.86	7.95	71.42	6.84	7.93
31a	$C_{23}H_{26}N_2O_4$	70.03	6.64	7.10	69.82	6.62	7.07
32a	$C_{22}H_{26}NO_2S$	61.38	6.09	6.51	61.62	6.11	6.53
33a	$C_{20}H_{23}NO_4S$	64.32	6.21	3.75	63.81	6.04	2.79
33b	$C_{20}H_{22}BrNO_4S$	53.10	4.90	3.10	52.88	4.88	3.08
54a	$C_{21}H_{22}N_2O_5$	65.96	5.80	7.33	66.01	5.73	6.98
54b	$C_{21}H_{21}BrN_2O_5$	54.68	4.59	6.07	54.86	4.47	6.02
5a	$C_{21}H_{24}N_2O_3$	71.57	6.86	7.95	71.44	6.67	7.78
5b	$C_{21}H_{23}BrN_2O_3$	58.48	5.37	6.49	58.09	5.15	6.29
1a	$C_{23}H_{26}N_2O_4$	70.03	6.64	7.10	69.85	6.36	7.02
2b	$C_{22}H_{25}BrN_2O_5S$	51.87	4.95	5.50	51.28	4.90	5.47
34a	$C_{16}H_{19}NO_5$	62.94	6.27	4.59	62.40	5.83	4.09
34b	$C_{16}H_{18}BrNO_5$	50.02	4.72	3.65	49.00	4.18	2.58
35a	$C_{16}H_{21}NO_4$	65.96	7.27	4.81	65.69	7.24	4.79
35b	$C_{16}H_{20}BrNO_4$	51.90	5.44	3.78	51.74	5.42	3.76
36a	$C_{15}H_{17}NO_5$	61.85	5.88	4.81	61.60	5.85	4.79
36b	$C_{15}H_{16}BrNO_5$	48.67	4.36	3.78	48.3	4.7	3.4
37a	$C_{21}H_{21}NO_6$	65.79	5.52	3.65	65.92	5.53	3.65

37b	$C_{21}H_{20}BrNO_6$	54.56	4.36	3.03	54.04	3.93	2.63
38a	$C_{20}H_{18}ClNO_5$	61.94	4.68	3.61	62.18	4.71	3.63
38b	$C_{20}H_{17}BrClNO_5$	51.47	3.76	3.00	51.12	3.57	2.76
39a	$C_{22}H_{23}NO_6$	66.49	5.83	3.52	63	6.0	3.2
39b	$C_{22}H_{22}BrNO_6$	55.47	4.66	2.94	55.52	4.66	2.94
40a	$C_{20}H_{19}NO_6$	65.03	5.18	3.79	64.83	5.16	3.77
40b	$C_{20}H_{18}BrNO_6$	53.59	4.05	3.12	53.37	4.03	3.10
41a	$C_{19}H_{16}ClNO_5$	61.05	4.31	3.75	60.80	4.29	3.73
41b	$C_{19}H_{15}BrClNO_5$	50.41	3.34	3.09	50.67	3.15	3.22
42a	$C_{19}H_{15}Cl_2NO_5$	55.90	3.70	3.43	56.15	3.55	3.47
42b	$C_{19}H_{14}BrCl_2NO_5$	46.85	2.90	2.88	46.89	2.61	2.83
43b	$C_{19}H_{14}BrF_2NO_5$	50.24	3.11	3.08	50.46	3.30	2.80
44b	$C_{19}H_{14}Br_2FNO_5$	44.30	2.74	2.72	44.30	2.69	2.51
45a	$C_{20}H_{21}NO_6$	64.68	5.70	3.77	64.98	5.84	3.57
46a	$C_{21}H_{23}NO_7$	62.83	5.78	3.49	63.02	5.67	3.44
47a	$C_{22}H_{25}NO_8$	61.25	5.84	3.25	61.06	5.82	3.24

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